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A METHOD OF DETERMINING THE SPECIFIC RENAL FUNCTIONS OF GLOMERULAR FILTRATION, MAXIMAL TUBULAR EXCRETION (OR REABSORPTION), AND "EFFECTIVE BLOOD FLOW" USING A SINGLE INJECTION OF A SINGLE SUBSTANCE

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METHODS of determining fairly specific renal functions are available at present.[1-3] Because of the complications and detail involved, their usage is limited essentially to the field of research. The following attempt at simplification of these procedures has meet with partial success and is presented in the interest of a wider clinical application of these newer and more useful renal function tests. No new concepts are involved, but in order to present our method, the underlying principles will be briefly reviewed from our perspective.

A substance which is excreted solely by being filtered through the glomerular membrane appears in the urine at a rate which is directly proportional to its concentration in the plasma water (Fig. 1). Inulin is an example of this type of compound. The ratio of its urinary excretion per minute to its plasma concentration is constant and may be expressed by the slope of the line indicating inulin excretion at varying plasma concentrations. This represents the inulin clearance, or glomerular filtration rate, in cubic centimeters per minute since it expresses the volume of plasma which contains the amount of inulin excreted in one minute. Inasmuch as the excretion of inulin is accomplished only by glomerular filtration, the slope of this line also represents the glomerular filtration rate; that is, the volume of plasma whose water is filtered by the glomerular membrane in one minute.

A substance which is not only filtered, but which also is excreted by the tubules, will have a higher rate of excretion than inulin. If the tubular excretion is proportional to plasma concentration, then the total excretion will likewise be proportional to plasma concentration, and the ratio $\frac{\text{urinary excretion rate}}{\text{plasma concentration}}$ will be constant; that is, the clearance will be constant and greater than the inulin clearance. This is generally true of diodrast in low concentrations (Fig. 2).

As their plasma concentration is increased, certain substances can be delivered to the tubules in quantities sufficient to saturate their tubular excretory

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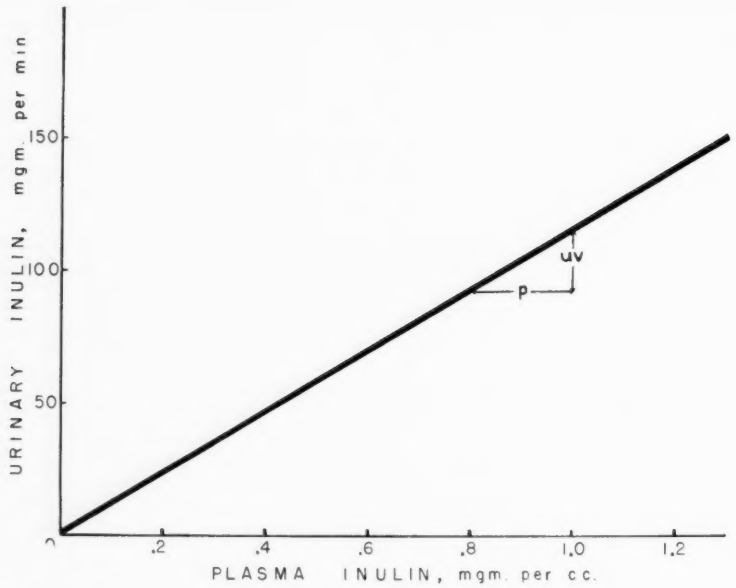

Fig. 1.—The excretion of inulin varies directly with its plasma concentration. The slope of this curve, $\frac{uv}{p}$, is constant and represents the plasma clearance of inulin. Since inulin is excreted solely by filtration, the value $\frac{uv}{p}$ measures the rate of glomerular filtrate formation.

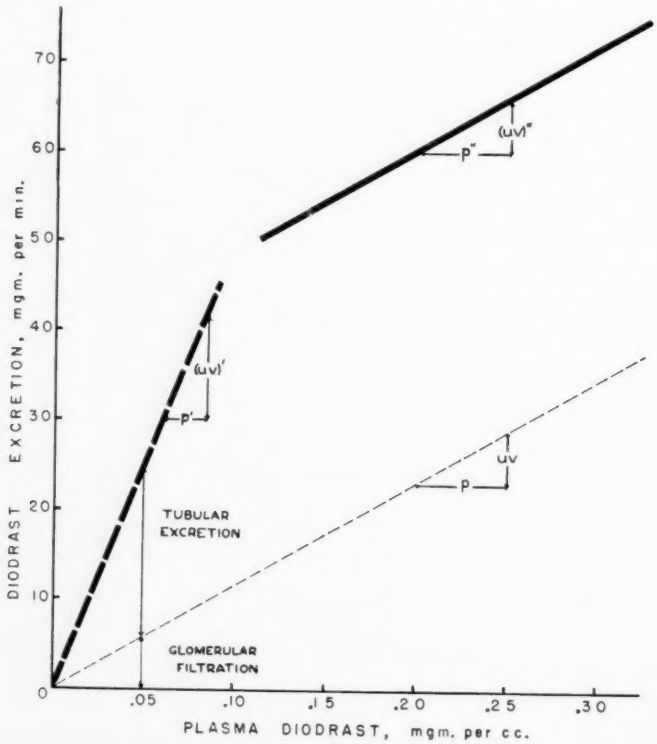

Fig. 2.—For diodrast at low plasma concentrations (heavy dashed line), $\frac{(uv)'}{p'}$ is constant and represents the diodrast plasma clearance. Since diodrast is excreted by both glomeruli and tubules, the contribution of each may be found if filtration is determined by simultaneous measurement of the inulin, or similar clearance.

For diodrast at high plasma concentrations, tubular excretion is constant at a maximum (Tm), while glomerular excretion continues to increase with increasing plasma concentration. The total excretion in this range (solid line), therefore, parallels the filtration curve, so that $\frac{\Delta\,(uv)''}{\Delta\,p''} = \frac{uv}{p}$ and again measures the glomerular filtration rate.

mechanism. In this case their tubular excretion rate may reach a constant maximum value. The *total* urinary excretion of such substances continues to increase as the plasma level is increased, but only because larger amounts are being filtered. Diodrast, above a plasma total concentration of about 15 mg. per cent, behaves in this fashion and exhibits a constant maximal tubular excretion rate referred to as its Tm (Fig. 2).

These three functions—glomerular filtration rate, diodrast clearance, and maximal diodrast tubular excretion (or Tm)—are usually determined by the simultaneous and sustained administration of inulin and diodrast. It may be readily shown theoretically that these functions can be determined by the use of diodrast alone—inulin being unnecessary for the following reason: Since the curve of urinary diodrast excretion in the range of high plasma levels is a constant distance above the curve of diodrast filtration, the two curves are here linear and parallel, and the slope of the curve for total excretion, therefore, again equals the glomerular filtration rate (Fig. 2). Furthermore, the distance the total excretion curve is above the filtration curve represents the maximum tubular excretion or Tm and can be read on the y axis by extrapolation. Data for these determinations can be obtained by a single intravenous injection rather than by a continuous intravenous infusion.

PLAN OF EXPERIMENTS

Experiments were designed to test the practical validity of this theorem. A single injection of diodrast (or para-amino-hippurate) was given and successive clearance periods obtained as the plasma concentration fell. Inulin was also included in the injection, in order to compare the simultaneous average inulin clearance with the glomerular filtration rate as determined from the curve of diodrast excretion. The following procedure was employed:

Preliminary to, and during, the test a large water intake was provided in order to maintain an adequate, approximately constant urinary flow and extracellular fluid volume. After a blood blank was withdrawn, the substance selected (here diodrast or para-amino-hippurate) was injected intravenously together with inulin in a conveniently small volume of solution. The amounts given were chosen to approximate the desired concentration in a volume of fluid whose mass is equal to 0.25 times body weight. The injection was given conveniently by gravity infusion in five to thirty-five minutes, the rate being controlled to minimize any alterations in blood volume or blood pressure. (Injection of diodrast in less than ten minutes has been noted to produce temporary depression of the diodrast clearance.)

Collection of urine was begun usually twenty-five to forty-five minutes after starting the intravenous injection. Three to twelve (average, seven) urinary samples were obtained, usually successively, each representing periods of nine to forty-six (usually ten to twenty-five) minutes. The usual technique for collection[2] was followed, using a large indwelling catheter and three successive quick rinsings of the bladder with 20 c.c. of saline, timing the end of rinsing with an accuracy within five to fifteen seconds.

Three or more blood samples were drawn at intervals to establish curves of plasma concentration. Duplicate or triplicate analyses of the heparinized blood plasma and of the diluted urine samples were made in the usual manner.[1-3] The plasma concentrations corresponding to two and one-half minutes before the mid-point of each collection period were read from the curve of log plasma concentration plotted against time. In the case of diodrast or para-amino-hippurate, these values were corrected for the water content of plasma and for protein binding, being expressed as milligrams of unbound material per cubic centimeter of plasma water. The average urinary excretion rates corresponding to each of these values were determined by dividing the total excretion of material in milligrams for each period by the length of the period in minutes. The excretion rates were then plotted as ordinates against the plasma concentrations as abscissas.

To all data in the region of maximal tubular function, a straight line was applied by the method of least squares. (This also can be done fairly accurately by inspection.) The selection of points can usually be made if it is remembered that the curve turns toward the origin at low values. The slope of the line obtained represents the average glomerular filtration rate in cubic centimeters per minute, and the extrapolated intercept on the y axis indicates the Tm in milligrams per minute. While this is termed a "graphic" method, the values also can be obtained readily from the formula for the line derived by the method of least squares. (The Tm is the value for y where $x = 0$, and the glomerular filtration rate is $\frac{dy}{dx}$.)

If urinary collections are continued, data may be obtained for low plasma levels. Here the slope of the curve increases to approach a straight line running through the origin. The slope of this line represents the "effective renal plasma flow."

RESULTS

Comparisons have been made in fourteen subjects in the following manner: (1) The glomerular filtration rate (GFR), derived by the aforementioned "graphic" method from diodrast or para-amino-hippurate excretions, was compared with the average of the simultaneous inulin clearance determinations (C_I) for each subject. (2) From the conventional calculations based on simultaneous excretions of diodrast (or PAH) and inulin, Tm's were obtained, and the average of these Tm's for each subject was compared with the "graphic" determination of Tm from diodrast (or PAH) excretion values alone. (3) When sufficiently low plasma levels were reached, the graphic and calculated "effective renal blood flow" were compared. (4) In several cases the modified procedure for the "graphic" method followed or preceded the "standard" method of maintaining continuous infusion at approximately constant plasma level, and the consecutive results were compared.

Representative curves are shown in Fig. 3, illustrating variation in grouping of points, self-depression of Tm due to too rapid injection, and clearances at high and low plasma levels of diodrast and PAH. The italicized values are

those calculated from the simultaneous inulin determinations. The comparisons are summarized in Table I. Of special significance are columns 5 and 6 and 10 and 11. Columns 5 and 6 indicate an average difference between methods of determining filtration rate of 8 c.c. per minute or 13 per cent, regardless of sign.

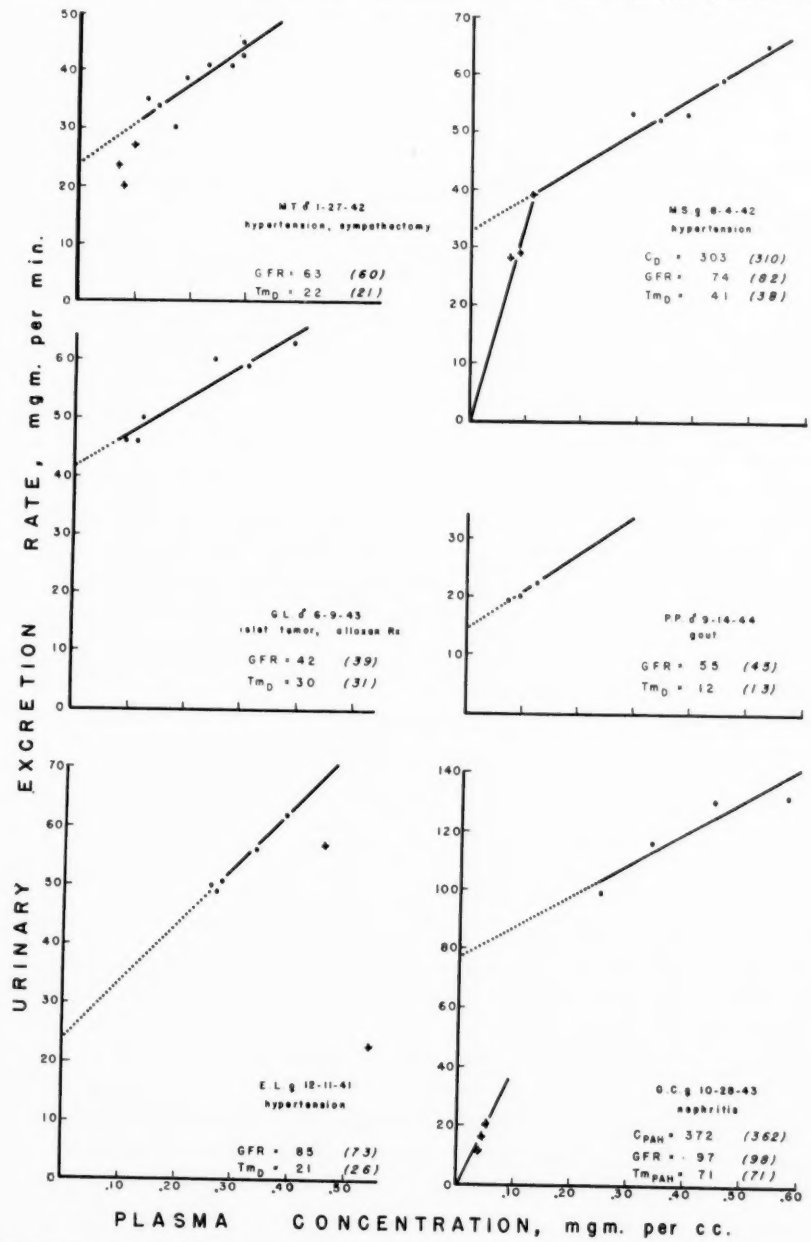

Fig. 3.—Representative experiments showing determination of renal functions by single injection technique. Simultaneous values as determined by standard technique are indicated in italics. (Data indicated as + do not apply to determination of glomerular filtration rate, (GFR) or Tm. Discussed in text.)

TABLE I. SUMMARY OF COMPARISONS OF SIMPLIFIED METHOD WITH STANDARD METHOD OF DETERMINING GLOMERULAR FILTRATION AND TM (DISCUSSED IN TEXT)

1	2	3	4	5	6	7	8	9	10	11
SUBJECT	GLOMERULAR FILTRATION RATE (SIMPLIFIED METHOD)	AVERAGE INULIN CLEARANCE	AVERAGE DEVIATION OF INULIN CLEARANCES	DIFFERENCE BETWEEN METHODS (COLUMNS 2-3)		Tm (SIMPLIFIED METHOD)	AVERAGE Tm (STANDARD METHOD)	AVERAGE DEVIATION OF Tm	DIFFERENCE BETWEEN METHODS (COLUMNS 7-8)	
	C.C./MIN.	C.C./MIN.	PER CENT	C.C./MIN.	PER CENT	MG./MIN.	MG./MIN.	PER CENT	MG./MIN.	PER CENT
J. M.	81	97	6	−16	−16	35	27	7	8	30
D. M.	54	64	4	−10	−16	30	26	4	4	15
L. W.	89	86	5	3	3	35	37	5	−2	− 5
E. L.	85	73	5	12	16	21	26	7	−5	−19
M. T.	63	60	9	3	5	22	21	9	1	5
J. D.	44	33	9	11	32	13	16	6	−3	−19
R. N.	56	63	16	− 7	−11	23	19	13	4	21
J. S.	52	63	5	−11	−21	32	30	5	2	7
J. P.	89	80	3	9	11	44	46	2	−2	− 4
C. M.	70	66	3	4	6	28	29	4	−1	− 3
M. S.	74	82	5	− 8	−10	41	39	5	2	5
G. L.	42	39	10	3	8	30	31	4	−1	− 3
G. C.	97	98	3	− 1	− 1	71*	71*	4	0	0
P. P.	55	45	12	10	22	12	13	6	−1	− 8
Average	68	68	7	8†	13†	31	31	6	3†	10†

*Para-amino-hippurate. All others are diodrast iodine.
†Regardless of sign.

This is to be contrasted with the average of the average deviations for the several inulin clearances in each experiment (column 4), indicating an average variation of 7 per cent in the determinations by the standard method itself. For the Tm comparisons, the average difference between the standard and the simplified method was 3 mg. per minute or 10 per cent, neglecting sign (columns 10 and 11), while by contrast the average of the average deviations of the standard Tm calculations was 6 per cent (column 9). The identity of the averages of the fourteen values for the two methods, both for filtration rates and for Tm, is, in view of the small number of cases, largely a prejudicing coincidence and does not argue for the reliability of the modified method in the individual case.

DISCUSSION

Errors.—This modification shares with all methods of measuring renal excretory function the errors arising from possible misunderstanding of the mode of excretion of the substance being studied, particularly in conditions of disease. Sampling errors are slightly greater than in the standard techniques, because of shorter rinsing, but are probably well within the analytical errors common to both methods.

There are, in addition, three errors introduced by the use of a changing plasma concentration rather than a sustained one. The first is that a representative sample of the mean plasma concentration may not be obtained. If the linear or the logarithmic rate of change of plasma concentration should happen to be constant, then the mean concentration can be read on a suitable graphic plot from the mid-point in time, or calculated from the values at the beginning (P_1) and end (P_2) of the period. Where the plasma concentration fall is linear, the mean concentration is equal to $\frac{P_1 + P_2}{2}$. Where the logarithm of concentration falls in a linear manner (as is the case with inulin and approximately so with diodrast at high plasma levels) the mean concentration is $\frac{P_1 - P_2}{2.302 \log \frac{P_1}{P_2}}$.[2]

For any relationship of plasma concentration and time, the true mean concentration can be arrived at by determining the entire area under the concentration curve for the period, either analytically or planimetrically, and dividing by the length of the period. In forty-two periods a comparison was made of the values as calculated by these three methods: from mid-point in time, by logarithmic plot, and by planimetric measurement. The differences between the values so derived (Table II) did not fall in any consistent direction and were less than the errors inherent in the remainder of the procedure; therefore, the simple "mid-point" value was taken as representative of the mean plasma concentration.

The second error is due to the fact that urine reaching the bladder is derived from plasma a short while previously, and, therefore, does not correspond to the simultaneous plasma concentration. This anachronism is due to

TABLE II. DIFFERENCES IN "MEAN PLASMA CONCENTRATION" AS OBTAINED BY (A) ARITHMETIC MEAN (B) LOGARITHMIC MEAN (C) PLANIMETRIC MEAN VALUES, ALL EXPRESSED AS PER CENT OF VALUE

COMPARISON	AVERAGE DIFFERENCE	MAXIMUM	MINIMUM
A-B	0.5	0.9	−0.8
A-C	0.4	0.6	−0.9
B-C	0.7	1.5	−0.5

a delay in the passage of urine through the kidney and ureters to the bladder, which is relatively greater than the circulatory delay in the passage of blood from the kidney to the arm vein (if venous blood is used). This time lag is variable and was found by Smith and co-workers[1] to average about two and one-half minutes in man. If the plasma concentration is constant no error results, but if the plasma level is rising or falling some correction should be considered. Applying a correction of two and one-half minutes to a curve of falling concentrations produces higher values for mean concentration, and therefore lower clearances, than if no correction were made. Using the conventional method of calculating clearances, but with a falling plasma concentration curve, the average decrease in clearance in thirty-one determinations was 2 per cent (the maximum, 4.3 per cent; the minimum, 0.8 per cent) when a two and one-half minute correction was applied. Conventionally calculating Tm from inulin and diodrast excretions based on a falling curve, a two and one-half minute correction resulted in even less change, since the Tm is derived by clearance differences. The average correction was −0.8 per cent; the range was from −3.0 per cent to +1.6 per cent. We have therefore applied the correction, concluding that the estimated anachronism of two and one-half minutes can be in error by 100 per cent without introducing significant error in result, except perhaps when very rapid changes in plasma level are occurring.

The third possible source of error arises in case of storage of material by the kidney, to be released during falling plasma concentration. Smith and co-workers[1] have concluded that there is no appreciable storage of inulin or of diodrast since clearances were the same whether obtained when plasma concentrations were rising or falling.

Technique of Test.—An important technical difficulty is presented in obtaining the proper range of blood levels. If renal function is high, or periods too long, too few points may fall within the usable range. On the other hand, if periods are too brief, inaccuracy in urine collection increases; or if renal function is too poor, the change in plasma level may not yield a sufficient scatter of points, unless the test be prolonged unduly. It is therefore imperative that some prior estimate of function be made in each case, as for instance by urea clearance, phenolsulfonphthalein, or concentration tests. When determinations of effective renal blood flow are also desired, it may take some time until sufficiently low plasma concentrations are reached in the case of large subjects, or in subjects with low renal function. In such cases the test may be interrupted and resumed if intermediate points are not desired.

Usefulness of Method.—In determining "effective renal plasma flow," this method offers an advantage over the standard one in that a check is provided on the constancy of renal extraction at different plasma concentrations. In renal disease the demonstration of a linear relation between excretion and plasma concentration of diodrast (or PAH) may be necessary before the clearance can be considered to represent "effective renal plasma flow."[4]

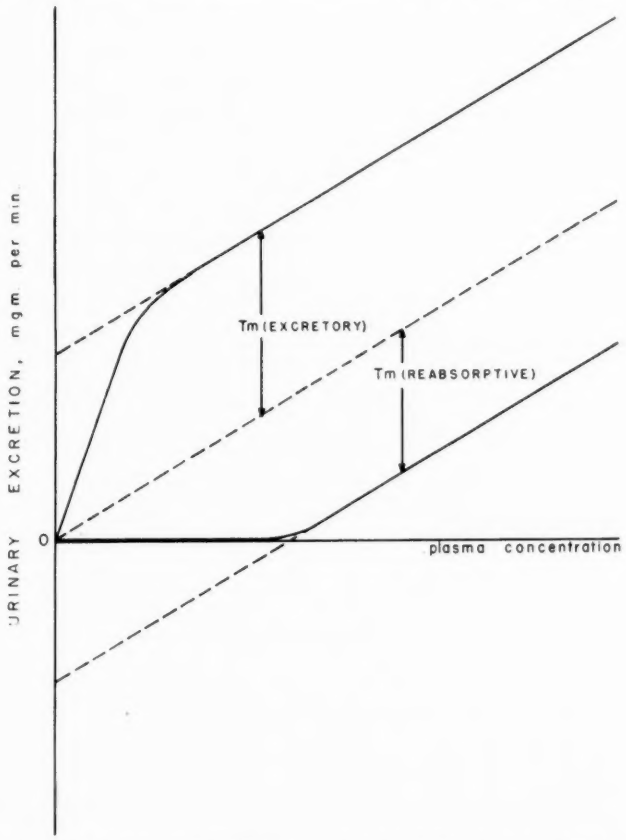

Fig. 4.—Various substances exhibiting a maximal tubular transport (Tm), either excretory or reabsorptive, may be used to determine the glomerular filtration rate as well.

By plotting excretion rates against the *total* concentration in the water of the plasma rather than against the concentration of *free* diodrast (or PAH), the Tm value will be unchanged, but the value for glomerular filtration rate will be low by a factor equal to the average per cent of material bound by protein. Smith and associates[3] have, in fact, adopted this method to enable them to check the degree of protein binding of PAH.

It is inaccurate to rely upon this method to estimate filtration fractions or to derive other hemodynamic ratios, for not only are the determinations of "effective blood flow" and glomerular filtration not made simultaneously, but the lessened accuracy of the determinations may yield disproportionate inaccuracy in any calculation involving ratios of the values obtained.

The fact that the calculations and analytic detail have been simplified should not suggest that the procedure has achieved true simplicity. We have not had a sufficient experience to predict whether it can be developed to a point where all the pitfalls of the more accurate clearance studies can be consistently avoided.

The method is applicable not only to diodrast, but also to any substance which exhibits a maximal tubular transport, either excretory or reabsorptive (Fig. 4). Data from the literature on hippuran, glucose, and ascorbic acid, when replotted by our method, finds agreement with the original calculations (Table III). Because the analysis of para-amino-hippurate is within the range of many clinical chemical laboratories, this substance is particularly suited to the method. The ideal substance is, however, still to be found, for it must possess the attributes of being nontoxic, not metabolized, not stored, nor rapidly excreted via other routes, must not appreciably or variably be bound to protein, must be readily subject to analysis, and its normal Tm should be large in relation to the error of analysis. With a substance possessing these attributes, a widespread clinical application of this simplified procedure may be feasible.

TABLE III. COMPARISON OF SIMPLIFIED METHOD WITH STANDARD METHOD OF DETERMINING GLOMERULAR FILTRATION AND TM
(Data from the literature; graph drawn by inspection)

		SIMPLIFIED METHOD		STANDARD METHOD			
SUBSTANCE	SUBJECT	GLOMERULAR FILTRATION RATE C.C./MIN.	Tm MG./MIN.	CREATININE CLEARANCE C.C./MIN.	INULIN CLEARANCE C.C./MIN.	Tm MG./MIN.	REFERENCE
Hippuran	Dog (3 experiments)	65	7.5	65.7		7.6	5 (Table III)
Diodrast	Dog	43	13.2	45		11.9	5 (Table II)
Glucose	Dog	117	292	115.2		268	6 (Table I)
Glucose	Dog	80	215	81.5		220	7 (Table I)
Ascorbic acid	Man (6 experiments)	130	2.5		124.2	2.17	8 (Table I)

Other Simplified Methods.—Other workers have reported on simplifications in the determination of these renal functions. The method of Findley and coworkers,[9] which assumes that the per cent of injected diodrast excreted in the urine in thirty minutes is a measure of Tm, has no physiologic basis. Applying his method to data determined by standard procedure has yielded errors of several hundred per cent.

Foà and Foà have performed clearances during falling plasma concentrations.[10] Our studies establish the validity of such procedure and offer further simplifications.

Earle and Berliner recently have suggested[11] that a sustaining infusion can be given to maintain a constant blood level after equilibrium has been established, when the rate of infusion can be taken to indicate excretion in place of the urinary collections, thus obviating the need of catheterization. How one

establishes the proper level of infusion other than by approximation is not indicated. It is not feasible to perform "spot" blood analyses during the test, and slow drifts in plasma concentration of insignificant degree may be produced by infusions at rates which are considerably different from the rate of urinary loss. For example, an infusion of 20 per cent more inulin than is being excreted will take twenty minutes to increase the plasma concentration by 2 per cent under conditions where plasma level is 100 mg. per cent, the total inulin in the body is 15 Gm., and the rate of excretion is 75 mg. per minute. If extracellular fluid volume remains constant over periods long enough to provide accuracy, then the procedure may have great practicality.

Newman and associates[12] have proposed that the rate of change in plasma level of mannitol after equilibrium has been established can be taken to yield the rate of excretion. We had endeavored to establish this possibility for inulin and diodrast. Disappointingly, we found that the theoretic volume of solution (obtained by dividing the amount of material remaining in the body by its plasma level) continued to rise and did not attain equilibrium during the several hour course of the experiment. Mannitol appears to equilibrate sooner than inulin and/or high concentrations of diodrast, but data establishing the correctness of the assumed equilibration do not appear in the literature. If the volume of distribution could be as reliably known, or held constant, in the case of substances in plasma concentration high enough to exhibit a Tm, then the mathematic derivatives of Tm without urinary collection may become possible. In this method of approach also, a small percentage change in volume of distribution accounts for an amount of test material which is large in proportion to the rate of excretion, especially at high plasma levels.

SUMMARY

Certain specific renal functions may be determined with sufficient accuracy for clinical purposes by blood and urine analyses after a single intravenous injection of one of several substances.

The theoretic implications and the present practical limitations of the method are discussed.

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RADIOACTIVE PHOSPHORUS, P^{32}: A SIX-YEAR CLINICAL EVALUATION OF INTERNAL RADIATION THERAPY

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FOR nearly half a century roentgen radiation has been the most effective palliative treatment for the more chronic leucemic states and allied hematologic dyscrasias, having been first used by Senn in a case of lymphatic leucemia in 1903.[1] There are, however, distinct and obvious limitations in the clinical application of roentgen-ray therapy as well as of radium and other naturally occurring radioactive elements. These have been utilized more or less effectively in medicine by way of external application, but these elements have proved dangerous when deposited within the body as internal therapy. With the development of the cyclotron by Ernest Lawrence, both physicist and biologist were provided with a tool by means of which a wide range of important hitherto stabile chemical elements in nature might be rendered temporarily instabile and radioactive for a limited period of time, thus opening up entirely new fields of scientific exploration and control.

In medicine, the diversion of small quantities of selected isotopes essential to normal body metabolism has made possible important tracer element studies, as well as selective internal radiation therapy. Very recently the provision for utilizing the government-owned uranium piles as a source of some of these biologically significant radioactive isotopes is permitting the more rapid development of this field of knowledge through a much greater potential supply of the desired elements.

Of the artificially produced isotopes, radioactive phosphorus (P^{32}) has been studied most extensively to date as a means of internal radiation therapy. It became evident following the earliest observations in animals that this isotope might be used advantageously in certain human hematologic dyscrasias. Clinical reports evaluating radioactive phosphorus as a therapeutic agent have been made by Low-Beer, Lawrence, and Stone,[2] Erf, Tuttle, and Lawrence,[4] Fitz-Hugh and Hodes,[5] Kenney,[6] Craver,[7] Warren,[8] Hall, Watkins, Hargraves, and Griffin,[9] Erf,[10, 11] and Hoster and Doan.[12] Reinhard, Moore, Bierbaum, and Moore[13] have recently published a comprehensive and detailed summary of the

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basic concepts and accumulated clinical experience in this field as a background for the critical analysis of their own observations which renders any repetition of these details at this time unnecessary and inappropriate.

Studies in this laboratory and clinic were initiated in 1941* with radioactive phosphorus from the Radiation Laboratory at Berkeley and were continued with material from the Ohio State University cyclotron when it became functional in 1942. We are now regularly receiving radioactive phosphorus and other isotopes through a government contract from the Manhattan Project, Oak Ridge, Tenn. An analysis of the results to date in our first 100 patients treated with radioactive phosphorus P^{32} is presented in this communication (Table I).

TABLE I. DISEASES TREATED WITH RADIOACTIVE PHOSPHORUS

Polycythemia rubra vera	20
The leucemias	
Lymphatic	
Acute (leucosarcoma)	18
Chronic	11
Myelogenous	
Acute	1
Chronic	13
Monocytic	
Acute	9
Chronic	1
Hodgkin's syndrome	16
Metastatic carcinoma and sarcoma	5
Multiple myeloma	4
Mycosis fungoides	1
Exfoliative dermatitis	1
Total	100

MATERIALS AND METHODS

The base line laboratory studies on all patients included serial complete total white, red blood cell, and platelet counts, white cell differentials using the supravital technique, hemoglobin determinations, reticulocyte percentages, sedimentation rate, and packed cell volume. Repeated bone marrow examinations via sternal aspiration and the supravital technique were made. Determinations of the basal metabolic rate were done routinely, excluding any thyroid dysfunction by the iodine fractionation techniques of Curtis and associates.[14] Appropriate biopsies were performed as the basis for fixed section diagnoses in the carcinomatoses, Hodgkin's syndromes, and mycosis fungoides. Blood volume determinations, using Evans blue dye T-1824,[15] formed a part of the study in patients with polycythemia rubra vera. Complete hematologic studies were made daily in those patients being observed in the University Hospital. Those who were seen as out patients had the same determinations made at intervals of one to eight weeks, depending upon the acuteness or chronicity of the clinical course and the availability of the patient. Certified United States Bureau of Standards Trenner blood cell pipettes were used. The sedimentation rate was estimated by the Wintrobe method as modified for cell volume by Rourke and Ernstene.[16] A modification of the Dameshek method was used for platelet and reticulocyte determinations. Hemoglobin measurements were made by the oxyhemoglobin determinations on the Evelyn photoelectric colorimeter, which was standardized by the Van Slyke apparatus.

Radioactive Phosphorus.—The details of the cyclotron bombardment of the red phosphorus have been adequately considered elsewhere.[17] The phosphorus isotope was converted

*Through the courtesy of Dr. John Lawrence.

into dibasic sodium phosphate by the method described by Kamen.[13] The route of administration in the earlier studies was determined by the availability and purity of the material. To conserve P^{32} the intravenous route was used most frequently, this being more economical by approximately 30 per cent. The more recent radioactive phosphorus from the uranium piles

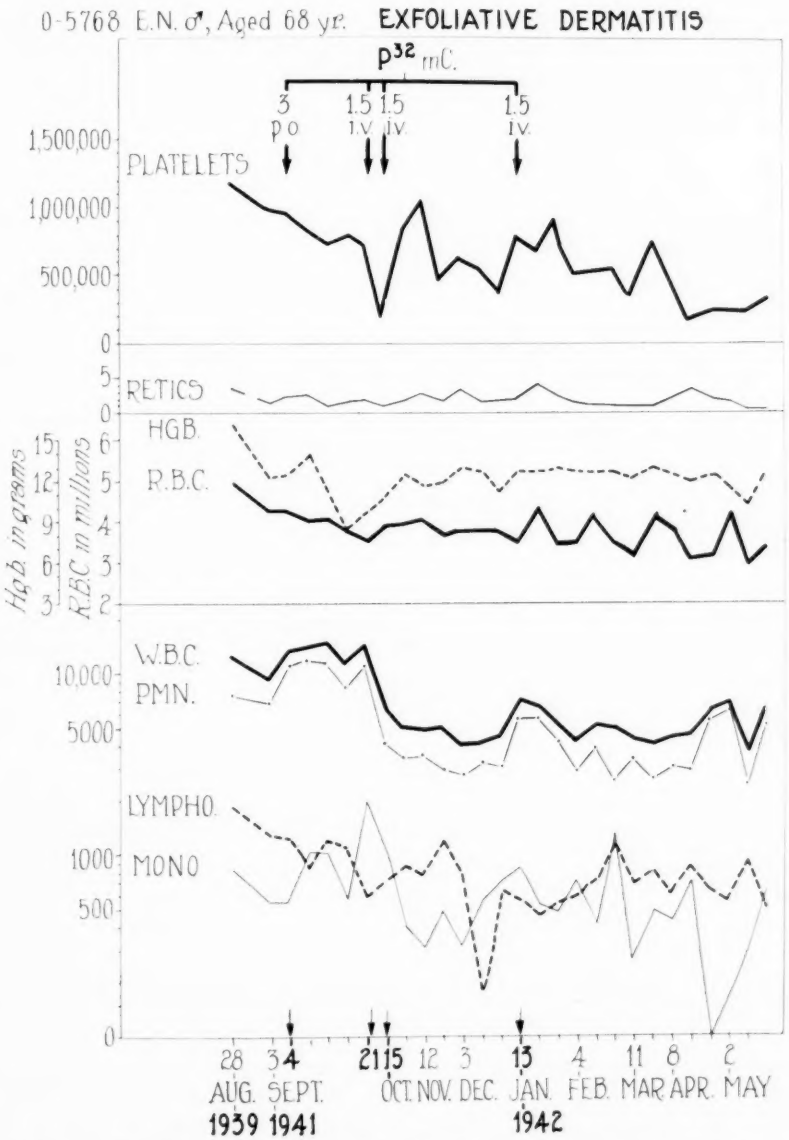

Fig. 1.—E. N., 68-year-old white man. Diagnosis: Exfoliative dermatitis of undetermined origin. Normal bone marrow and peripheral blood.

Under P^{32} therapy there was improvement in the skin lesions, with corresponding disappearance of the pruritus. Note the progressive depression and decline in the platelet, erythrocyte, hemoglobin, granulocyte, lymphocyte, and monocyte levels during and after the course of P^{32}. These hematologic changes fortunately re-equilibrated at subclinical levels but serve to warn against the too-free administration of radioactive isotopes where normal hematopoiesis may be primarily concerned.

at Oak Ridge has been supplied as potassium dihydrogen phosphate (KH_2PO_4), and, due to the relatively large amount of potassium, administration has been confined to the oral route.

The Geiger counter has been used in making the measurements of radioactivity.

Phosphorus is selectively deposited in bone and in the nuclei of rapidly multiplying cells. The radioactive and nonradioactive isotopes of phosphorus have identical chemical properties. Thus it is possible for the beta rays emitted by the radioactive phosphorus deposited at these sites to exert maximum inhibitory and destructive effects on adjacent marrow elements. The effect on normal marrow and peripheral blood is illustrated in Fig. 1, data on a patient with resistant exfoliative dermatitis who responded to small doses of radioactive phosphorus with definite but subclinical hematologic side effects.

POLYCYTHEMIA RUBRA VERA

In polycythemia rubra vera there is a characteristic *triune hyperplasia* of all marrow elements—myeloid, erythroid, and megakaryocytic. With the therapeutic means that have been previously employed in this syndrome, including venesection, phenylhydrazine, and various erythrotoxins, adequate control of the hyperplasia of all of the marrow elements has been unsatisfactory.

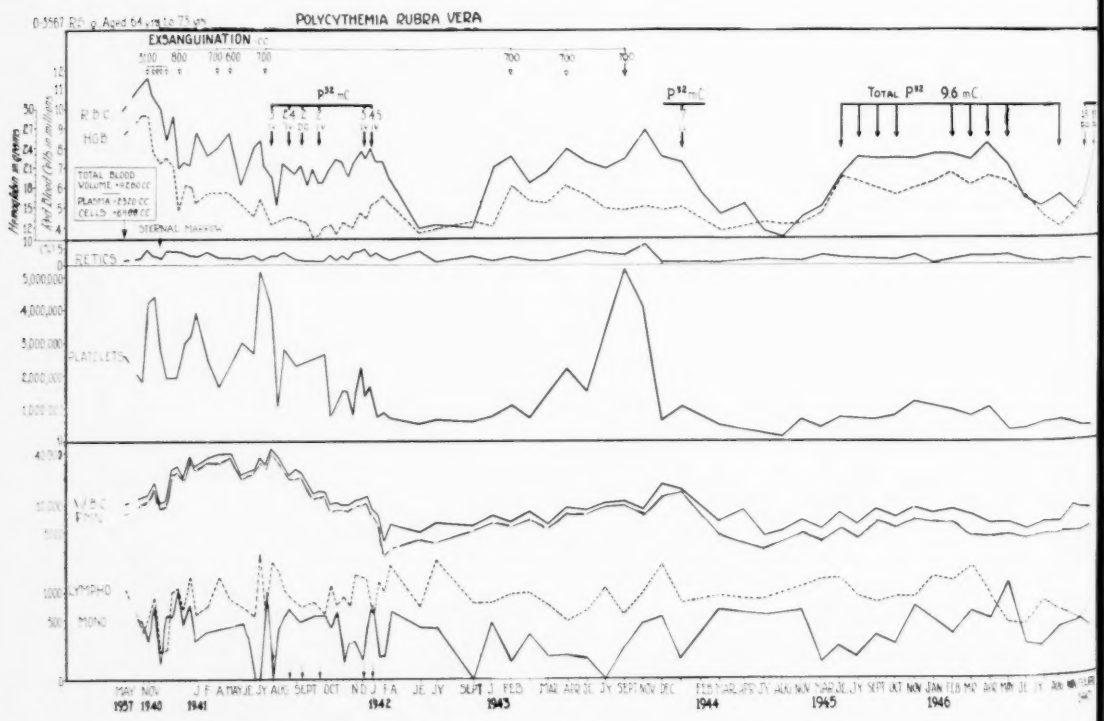

Fig. 2.—R. S., 73-year-old white woman. Diagnosis: Polycythemia rubra vera.

Venesection during the first four years of observation, while reducing the erythrocyte level, failed completely to control either the white blood cell or the platelet levels which actually rose steadily with accompanying clinical symptoms. The leucemoid picture was promptly controlled by P^{32}. Note the sharp rise in platelets following venesection in the period 1937 to 1941 and again in 1943, and the critical response to P^{32} on each occasion. Since 1941, three complete hematologic and clinical remissions have been induced with P^{32} therapy lasting respectively twelve months, seventeen months, and, currently, seven months. An excellent example of the control of the *triune marrow hyperplasia* with internal radiation therapy.

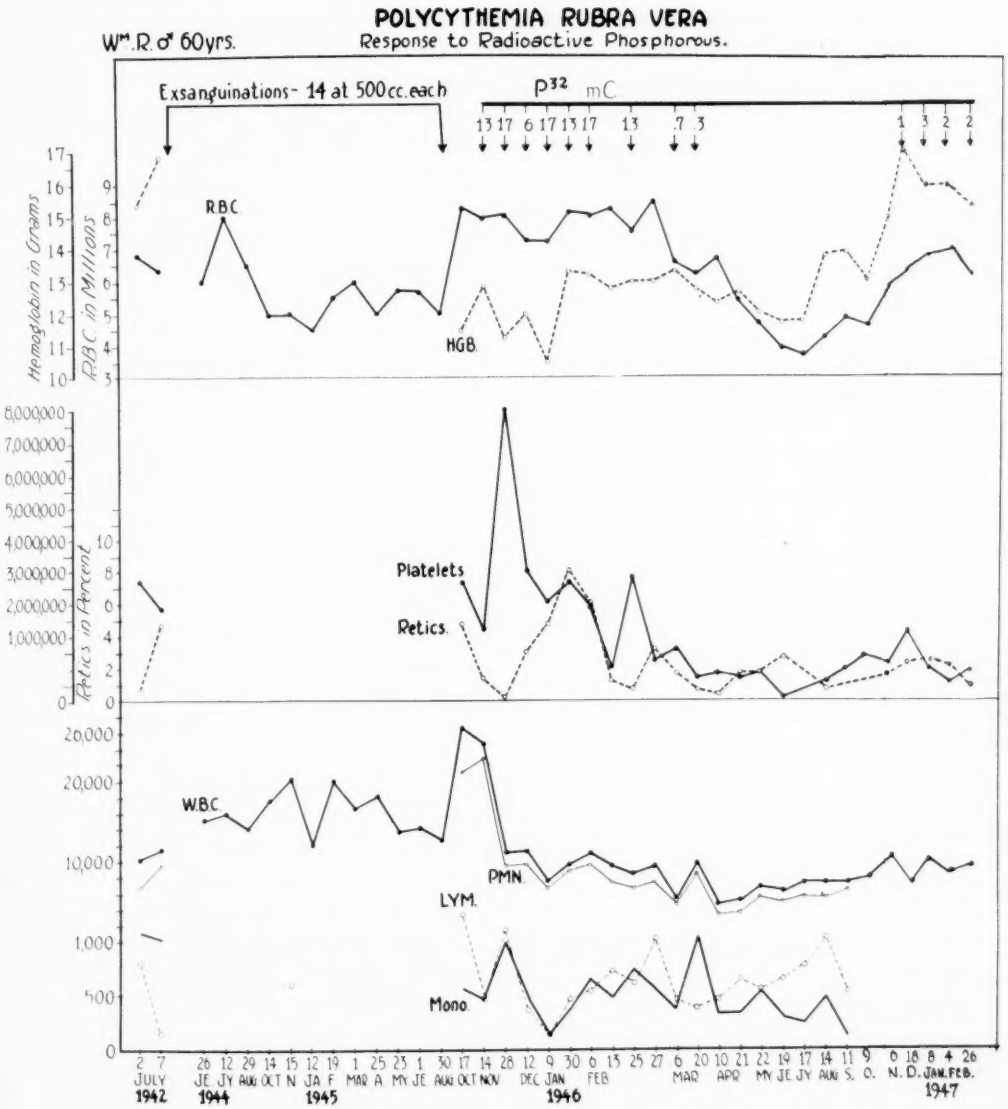

Fig. 3.—W. R., 60-year-old white man. Diagnosis: Polycythemia rubra vera. During the three months preceding the establishment of the diagnosis in 1942 this patient had both cerebral and coronary thromboses. Venesections and roentgen radiation together failed to maintain a satisfactory hematologic or clinical equilibrium during the next three years. A total of 10.6 mc. P^{32} administered in small doses over a four-month period induced a remission of one year. Second remission now being induced. Complete relief of the incapacitating vertigo and recurrent intravascular thromboses has been effected. Note the response of the platelets (8,000,000 per cubic millimeter) and granulocytes (26,000 per cubic millimeter) as well as erythrocytes (8,300,000 per cubic millimeter) to this therapy.

This is particularly true of the megakaryocytic hyperplasia and consequent potentially dangerous thrombocytosis. The development at times of hypoprothrombinemia, or the increased viscosity of the blood, plus an elevated level of circulating platelets, makes the opposite extremes of spontaneous hemorrhage or intravascular thrombosis potentially dangerous complications to be anticipated and avoided. When radioactive phosphorus is deposited in bone, the beta radiation is effective in controlling myeloid and megakaryocytic hyperplasia as well as erythroid hyperplasia. With the more effective control of the pan-marrow hyperplasia the incidence of complications is significantly lowered and longevity increased. The experience in this clinic is in agreement with the reports of others, that at the present time properly administered radioactive phosphorus is the therapy of choice for polycythemia rubra vera.

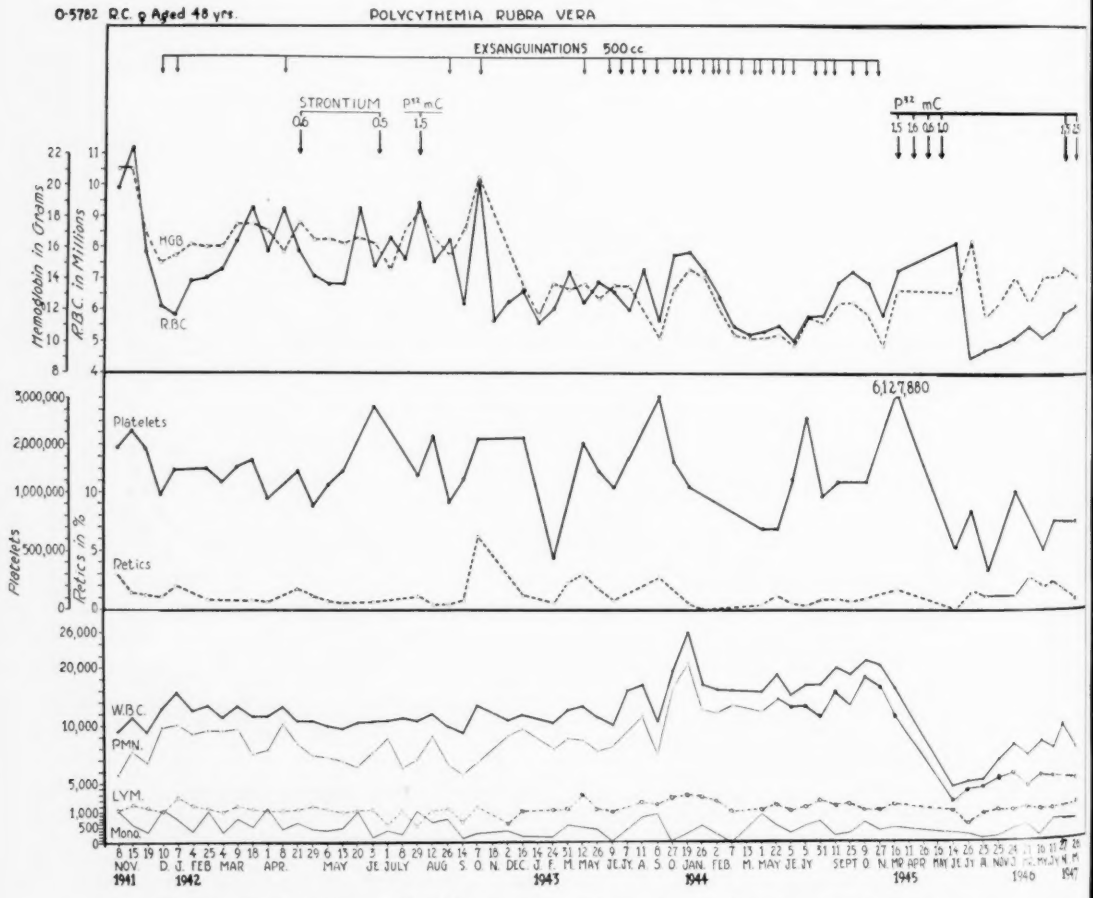
O-5782 R.C. ♀ Aged 48 yrs.
POLYCYTHEMIA RUBRA VERA
EXSANGUINATIONS 500 cc.
STRONTIUM
P32 mC
Hemoglobin in Grams
R.B.C. in Millions
HGB.
R.B.C.
Platelets
Retics
6,127,880
W.B.C.
PMN.
LYM.
Mono.
NOV. D J FEB MAR APR. MAY JE JULY AUG S O N DEC J F M MAY JE JY A S O JAN. FEB M MAY JE JY SEPT O N MR APR MAY JE JY A NOV J
1941 1942 1943 1944 1945 1946 1947

Fig. 4.—R. C., 48-year-old white woman. Diagnosis: Polycythemia rubra vera.

Small token doses of radioactive strontium and phosphorus in 1942 induced suggestive but not prolonged remission in this patient. Subsequent repeated venesections, while controlling the red blood cell and hemoglobin levels fairly satisfactorily, failed to influence favorably leucocyte and platelet levels, which reached 21,000 and 6,127,800 per cubic millimeter, respectively, by April, 1945. With four small divided doses of P^{32} therapy a remission lasting fifteen months was accomplished, which controlled effectively all marrow elements.

A total of twenty patients with this diagnosis, twelve men and eight women, have been treated in this series (Table II). The ages range from 28 to 73 years. Only two have expired during this period of observation, one from injuries received in an automobile accident and the other from carcinoma of the bladder. Excellent hematologic and clinical remissions lasting from five to nineteen months were effected in ten (Figs. 2 to 4). Seven patients showed partial responses. Four of these required venesection at various times in association with the P^{32} therapy. Two (Cases 7 and 11) had a fall in both white blood cells and platelets to a low normal before the red blood cells had shown a satisfactory response. In both of these instances venesection seemed indicated to bring the red blood cell level to normal since it was feared that further internal radiation therapy might cause a clinically significant granulocytopenia or thrombocytopenia. Thrombocytopenia, neutropenia, and anemia have been noted as complications of P^{32} therapy,[18] and the necessity for careful individualization of dosage cannot be overemphasized. Another case (Case 12) has been seen only at infrequent intervals over the past three years. Only when pruritus became severe was medical attention sought. P^{32} controlled the pruritus satisfactorily. In the limited dosage then available, however, little effect was observed on the hematologic picture. During the earlier years of this study the supply of radioactive phosphorus was very limited, and in the majority of instances where satisfactory remissions were not induced the P^{32} dosage was definitely inadequate. No patient with this diagnosis has developed a true irreversible leucemic, hematologic, or clinical picture in our experience, though a "leucemoid" blood picture has not been unusual.

LYMPHATIC LEUCEMIA

Ten of the eleven patients with chronic lymphatic leucemia (Table III) treated with P^{32} have expired. One is still under therapy (Fig. 5). Excellent clinical and hematologic improvement was obtained in three patients, one of whom (Fig. 6) had developed a marked hypersensitivity to roentgen radiation. Only moderate improvement was effected in three cases and slight improvement in two. In three patients no change in either clinical or hematologic status was apparent; two of these previously had become resistant to roentgen radiation. Three patients with intractable pruritus associated with leucemia cutis were relieved with P^{32} therapy, one of these having received no relief previously from spray roentgen radiation.

LEUCOSARCOMA

(LYMPHOSARCOMA CELL LEUCEMIA)

Fifteen of the eighteen patients with leucosarcoma treated in this series were children under 9 years of age (Table IV). In twelve cases temporary remissions, both clinical and hematologic, were induced, lasting from three days to three weeks; however, the leucemic process then became more resistant to radiation therapy and occasionally appeared actually to be accentuated (Fig. 7). All of these patients have expired.

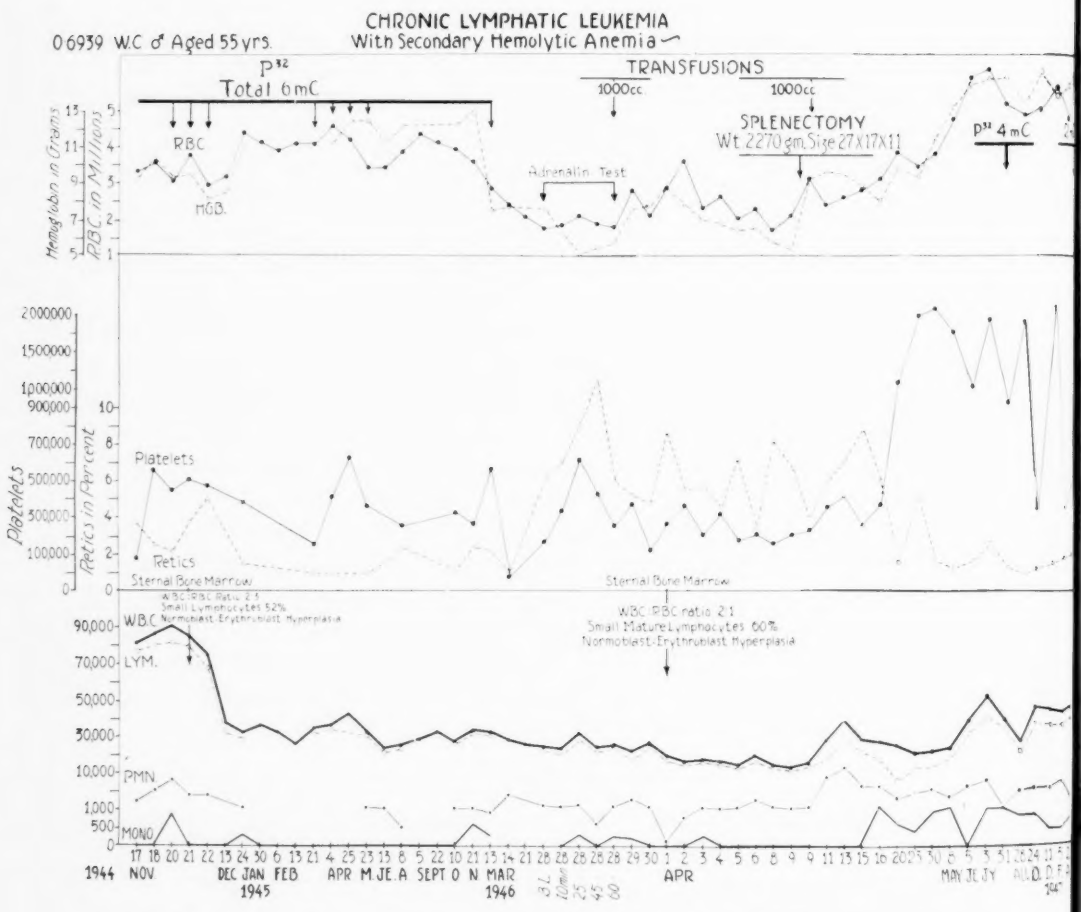

Fig. 5.—W. C., 55-year-old white man. Diagnosis: Chronic lymphatic leucemia.

Radioactive phosphorus reduced the white blood cells from 90,000 to 20,000 with a reciprocal rise in the circulating red blood cells and hemoglobin levels; the patient was asymptomatic for a sixteen-month period from November, 1944, to March, 1946, on this regime. Then there suddenly developed a subacute erythroclastic crisis with clinical jaundice and a rapid fall in red cells and hemoglobin during a three-week period. The reticulocytes became moderately elevated (6 to 11 per cent). The spleen increased rapidly in size but there was no corresponding enlargement of any of the regional lymph nodes, and the peripheral white cell and lymphocyte equilibrium showed no significant qualitative or quantitative alterations. The bone marrow revealed a W.B.C.:R.B.C. ratio of 2:1 with 60 per cent small mature lymphocytes present, but there was striking evidence of normoblastic and erythroblastic overcompensatory hyperplasia. There was no sign of excessive destruction of marrow elements by the radioactive phosphorus. The adrenalin test further supported a diagnosis of hypersplenism, and splenectomy was advised. Following splenectomy clinical and hematologic equilibrium was promptly re-established. This is an unquestionable example of secondary hypersplenism developing during the course of chronic lymphatic leucemia but unassociated with any generalized constitutional leucemic exacerbation. The remission continues with complete clinical recovery and return to full occupation as of June 1, 1947.

MYELOGENOUS LEUCEMIA

Fourteen patients with myelogenous leucemia have been treated with radioactive phosphorus (Table V). All were chronically ill from a clinical and hematologic point of view except one, a patient with an acute basophile granulocyte leucemia. The criteria for dosage included not only the maintenance of a satisfactory total white blood count, but also of adequate erythrocyte and hemoglobin levels. These latter objectives were satisfactorily accomplished in most instances.

Control of the splenomegaly in these patients proved to be much more difficult than the lowering of the peripheral white cell count (Fig. 8). Frequently roentgen radiation over the spleen had to be invoked to relieve the symptoms referable to the mechanical discomfort occasioned by the massive enlargement of this organ. More intensive P^{32} therapy was contraindicated because of peripheral blood and bone marrow findings.

In this limited series of cases of myelogenous leucemia, radioactive phosphorus has not proved to be as effective a therapeutic agent on the whole as roentgen radiation (Fig. 9). It is, however, a very useful therapeutic agent in those

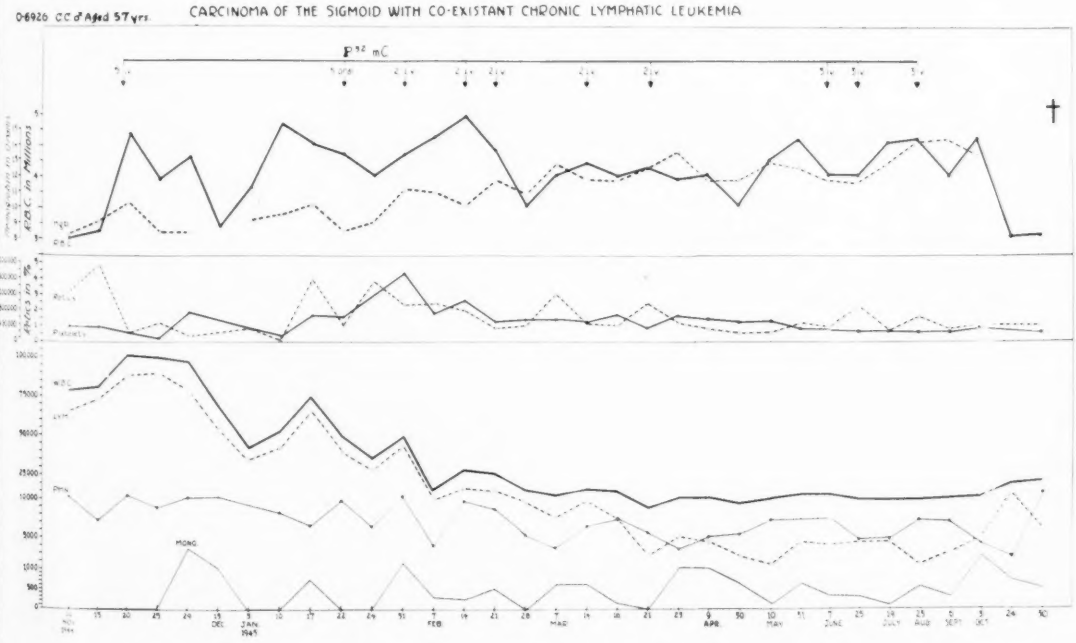

Fig. 6.—C. C., 57-year-old white man. Diagnosis: 1. Chronic lymphatic leucemia and 2. carcinoma of the lower bowel.

Medical attention was sought first by this patient for a lower bowel obstruction. Carcinoma of the sigmoid was diagnosed on biopsy. Coexistent lymphatic leucemia was found.

The leucemic process was brought under excellent control with radioactive phosphorus therapy, the white blood count, red cell count, and platelets being maintained near normal values. The carcinomatosis progressed and was the cause of death. At the time of death the leucemic process was under excellent control.

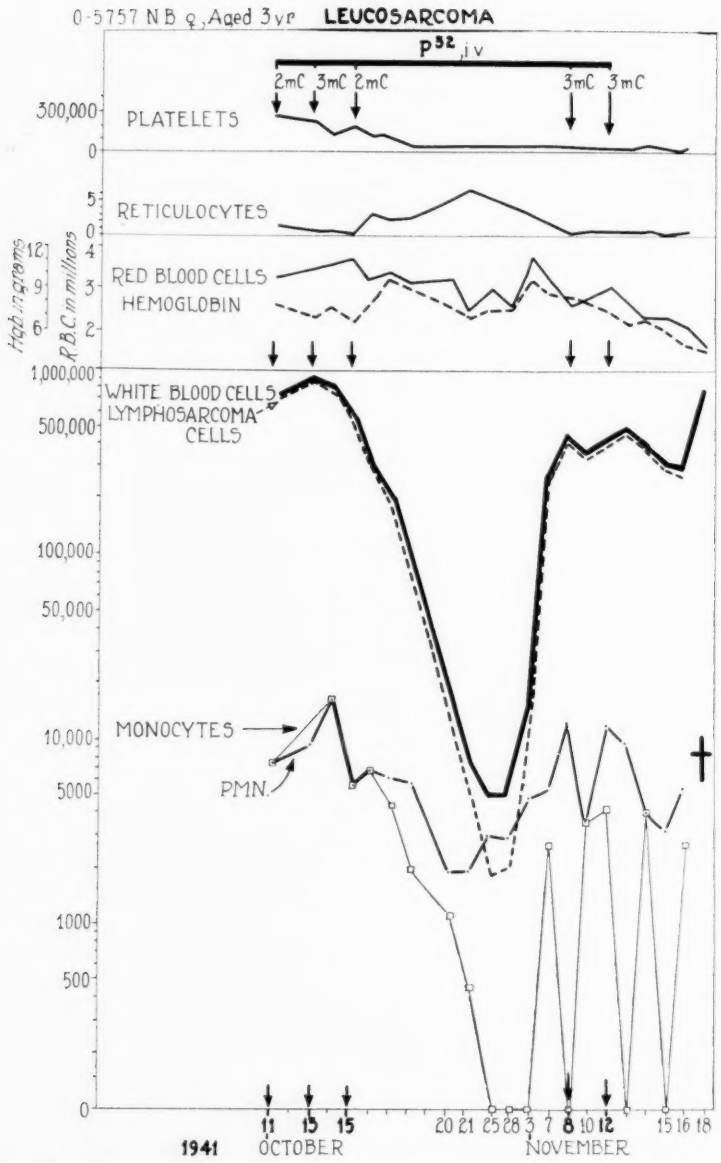

Fig. 7.—N. B., 3-year-old white girl. Diagnosis: Leucosarcoma.

With 7 mc. of radioactive phosphorus over a four-day period there was a precipitous drop in the white blood count from a high of 966,000 per cubic millimeter to 5,000, with a marked regression of the generalized lymphadenopathy and splenomegaly. This clinical and hematologic remission lasted only three weeks. The second course of P^{32} therapy failed to have any clinical or hematologic effect.

patients who develop an intolerance to roentgen radiation, with intractable and incapacitating constitutional reactions to even very small dosages, as was the case in three patients in this series.

MONOCYTIC LEUCEMIA

Eight of the nine patients with monocytic leucemia treated in this series were clinically acutely ill (Table VI). In the one chronically ill patient, mod-

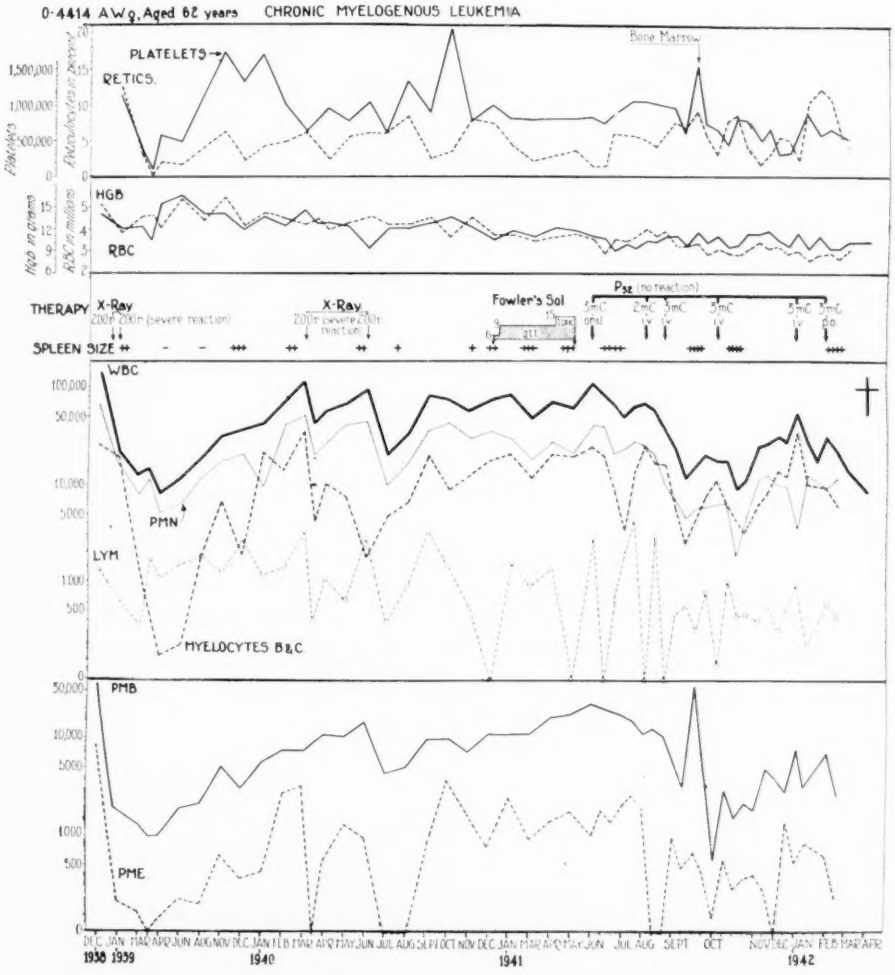

Fig. 8.—A. W., 63-year-old white woman. Diagnosis: Chronic myelogenous leucemia. The myelogenous leucemia in this patient responded initially to roentgen radiation with prompt reduction in the splenomegaly; however, she experienced severe radiation sickness following each treatment. A six-month trial of Fowler's solution failed to accomplish satisfactory results within the tolerance of the patient for arsenic. P^{32} was then instituted without any clinical reactions. The peripheral hematologic picture was quickly and adequately controlled. The splenomegaly, which was the source of most of the subjective complaints, continued to progress and became chiefly responsible for the terminal intestinal obstruction.

erate clinical improvement followed the use of P^{32} over a twenty-one-month period. In only three instances was a temporary favorable effect induced in those acutely ill, lasting from two to four weeks (Fig. 10).

HODGKIN'S DISEASE

Eleven of the sixteen patients with Hodgkin's syndrome treated with radioactive phosphorus in this clinic have been reported previously by Hoster and Doan.[12] In none of these was significant benefit from this form of therapy noted. There were several instances in which a dangerous depression in thrombocytes, erythrocytes, and leucocytes occurred incidental to the P^{32} therapy.

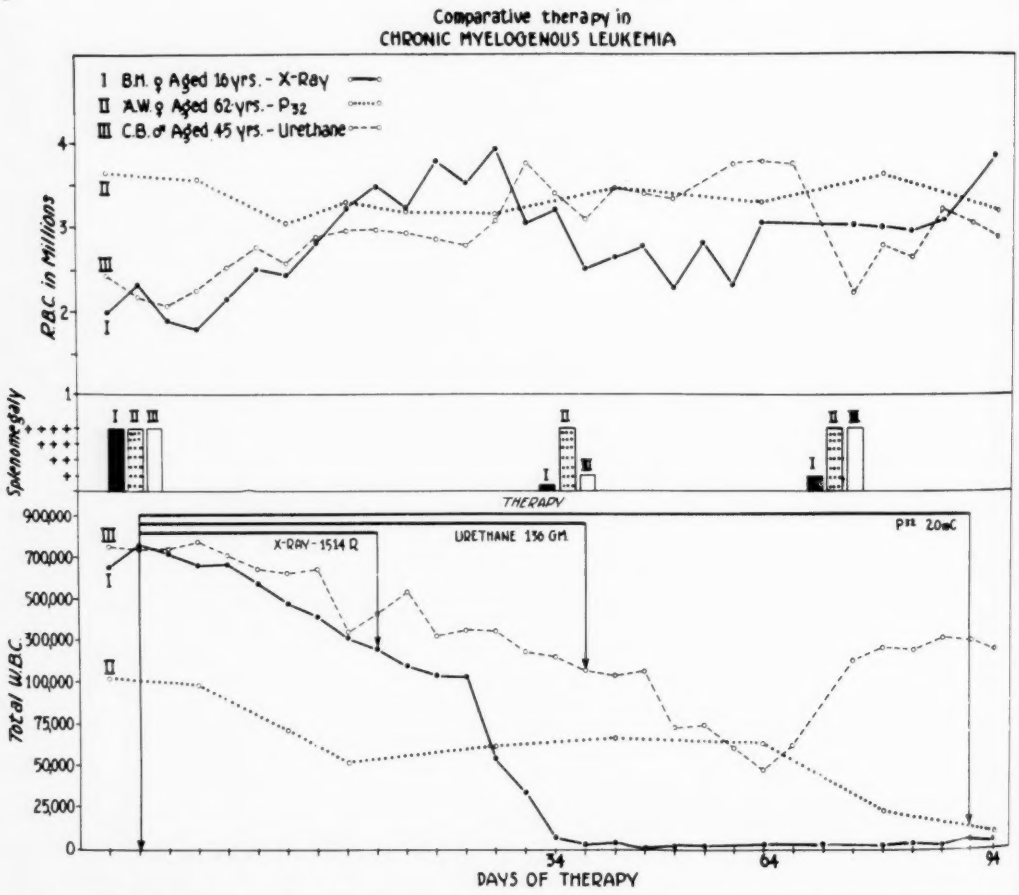

Fig. 9.—Comparative effectiveness of (*I*) external roentgen irradiation, (*II*) internal radiation with P^{32}, and (*III*) oral urethane chemotherapy in three patients with chronic myelogenous leucemia. Both spleen size and excessive circulating white blood cells were controlled most effectively by roentgen irradiation; the remission in this patient is continuing after six months. P^{32} reduced the elevated white count but had no effect on the splenomegaly. Urethane produced a transient clinical remission with a fall in the total circulating white cells and rapid decrease in size of the spleen. Upon discontinuing the daily urethane dosage there was an immediate rise in white count with concurrent re-enlargement of the spleen.

MISCELLANEOUS

A small group of patients (four with multiple myeloma, five having malignancies with metastases to bone, one with exfoliative dermatitis of undetermined etiology, and one with mycosis fungoides, Table VII) may be evaluated together. In the patients with malignancies involving bone, temporary relief from pain was obtained, lasting from two weeks to six months; however, no appreciable regression in the lesions could be recognized roentgenologically or clinically.

The cutaneous lesions, associated with a marked pruritus in one patient with exfoliative dermatitis, were significantly improved under P^{32} therapy; however, the bone marrow, which had been normal at the time therapy was started in this patient, reflected the depressant effect of the radioactivity and provided

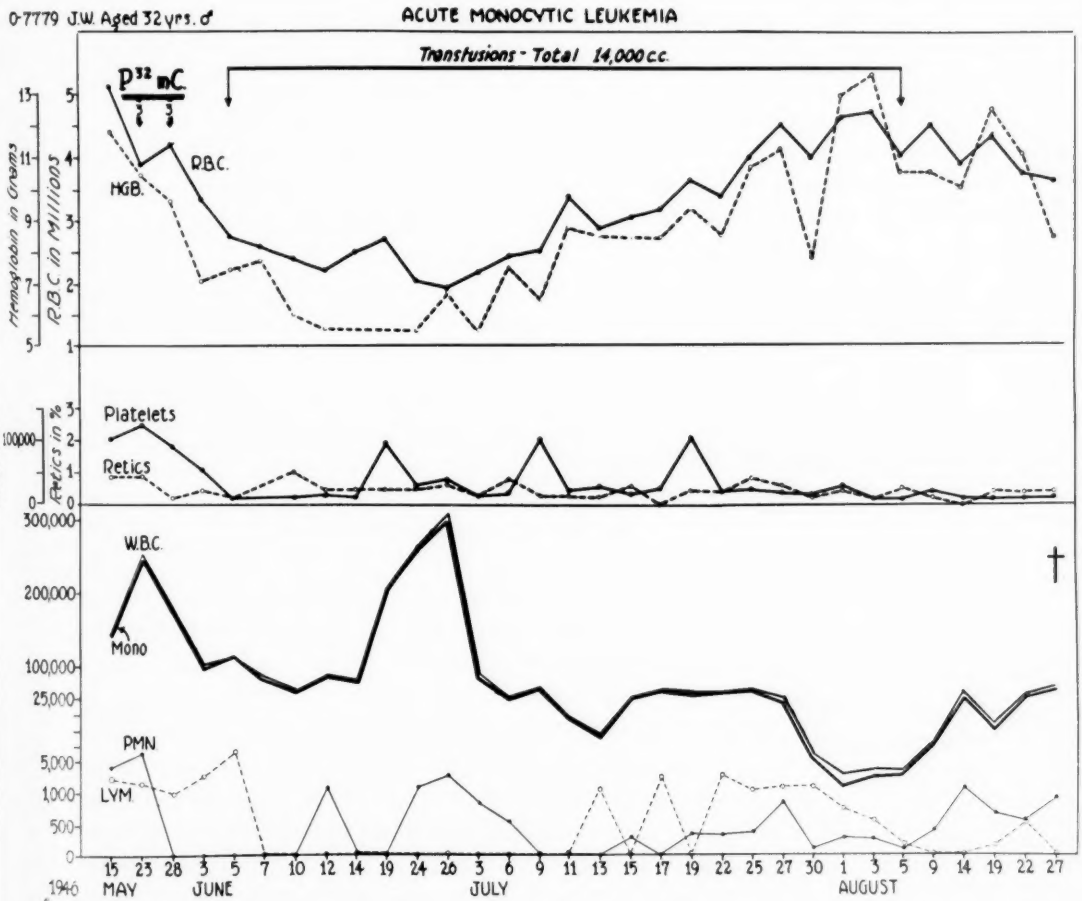

Fig. 10.—Following 6 mc. of P^{32} there was a fall in the white blood count from 253,000 to 88,000, during which regression it was still necessary to support the patient with repeated blood transfusions, and neither circulating reticulocytes nor platelets nor granulocytes reflected any regression in marrow invasion by monoblasts. The efficacy of any specific therapy under such circumstances is, of course, very questionable.

the chief criterion upon which the dosage tolerance was established. The patient with mycosis fungoides was under observation for two months. The course was steadily downhill, and he expired without receiving any apparent benefit from therapy.

Aside from the temporary relief of bone pain and pruritus, it is felt that the phosphorus radioactive isotope therapy had no significant effect on the course of the disease in any of these patients.

CONCLUSIONS

1. Radioactive phosphorus has been, in this clinic to date, of greatest value in controlling the clinical and hematologic manifestations of polycythemia rubra vera.

2. P^{32} is a valuable adjunct to other therapy in certain of the more chronic leucemic states, particularly in those patients intolerant of or resistant to roentgen radiation. The splenomegaly in chronic myeloid leucemia has frequently proved more resistant to P^{32} than to deep x-ray therapy.

3. Although internal radiation therapy may cause an occasional brief favorable effect in the acute leucemias, all too frequently P^{32} has appeared to accentuate the clinical acuteness of the leucemic process and has never proved permanently beneficial in our hands.

4. Radioactive phosphorus has failed effectively to control Hodgkin's syndrome in our clinic and may threaten the integrity of the marrow unless great care and discretion are observed in the dosages used.

5. The deep bone pain which characterizes metastatic malignancies to bone may be relieved by P^{32}. The evidence as to specific retardation of the lesions remains questionable.

6. Pruritus secondary to leucemia cutis, polycythemia rubra vera, and exfoliative dermatitis has been effectively controlled by radioactive phosphorus therapy in selected instances.

7. Our source of radioactive phosphorus until recently has been the University of California (Berkeley) and Ohio State University cyclotrons. The supply of isotopes has been correspondingly limited. The recent decision to release radioactive phosphorus, as well as other radioactive isotopes, from the uranium piles of the Manhattan Project for medical and biologic use will make this form of internal radiation much more readily available to clinical investigators. *It must be emphasized that the efficacy of this therapy depends upon "selective" cell destruction. There is a wide variance in individual susceptibility and tissue response. Extreme care in administering internal radiation therapy is therefore essential. Dosage must be carefully adjusted to each patient's needs and to his idiosyncrasies as determined individually. Our experience suggests small, well-spaced doses of radioactive phosphorus with frequent, careful, complete blood and bone marrow studies to insure the integrity of normal hematopoiesis during this treatment of human diseases.*

TABLE II. RADIOACTIVE PHOSPHORUS THERAPY IN POLYCYTHEMIA RUBRA VERA

TABLE II. RADIOACTIVE PHOSPHORUS THERAPY IN POLYCYTHEMIA RUBRA VERA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY: R.B.C. (PER C.MM.)	ONSET OF THERAPY: HEMOGLOBIN (GM. %)	ONSET OF THERAPY: W.B.C. (PER C.MM.)	ONSET OF THERAPY: PLATELETS (PER C.MM.)	P^{32} DOSAGE* (MC.)	MINIMAL COUNTS UNDER P^{32} THERAPY: R.B.C. (PER C.MM.)	MINIMAL COUNTS UNDER P^{32} THERAPY: HEMOGLOBIN (GM. %)	MINIMAL COUNTS UNDER P^{32} THERAPY: W.B.C. (PER C.MM.)	MINIMAL COUNTS UNDER P^{32} THERAPY: PLATELETS (PER C.MM.)	IMPROVEMENT OF SYMPTOMS	REMARKS
1	T. G.	64, W., M.	6.7	13.1	35,000	2,492,600	7.5 over 5 mo.	5.0	10.1	18,000	991,800	+++	Good remission for one year; treatment discontinued due to limited amount of P^{32}
2	L. J. S.	58, W., M.	(1) 6.9	12.6	37,000	2,603,104	16 over 9 mo. (o)	5.8	10.4	5,450	384,400	++	Initial therapy only moderately satisfactory, then a 14-month period of venesections; second course was effecting satisfactory control when patient was killed in automobile accident, 30 days after last therapy
			(2) 7.0	14.6	17,650	229,240	7 (o)	6.0	10.1				
3	E. W. S.	47, W., M.	6.1	10.2	18,000	3,622,600	2 (o)	5.1	9.7	10,300	1,840,320	++	Five-month follow up with good control clinically and hematologically; note W.B.C. and platelets particularly; no further studies
4	H. S.	66, W., F.	6.5	16.9	16,150	3,308,760	3 (o)	7.3	13.4	10,150	2,769,780	?	One small oral dose P^{32} only; insufficient therapy available
5	H. D.	55, W., M.	7.8	18.0	17,900	789,000	3 (o)					?	One small dose P^{32}; uncooperative
6	E. B.	40, W., M.	8.0	15.9	19,000	2,880,000	5.6 over 3 mo.	3.9	12.9	6,450	249,000	+++	Remission 8 months after initial course; has maintained satisfactory hemogram within normal values, with additional therapy as indicated
							3.6 over 8 mo.						
7	J. V.	28, W., M.	7.74	20.1	5,750	913,320	5.7 over 8 mo.	4.4	12.1	3,150	378,540	+++	Phenylhydrazine therapy for 9 months preceding P^{32}; Neutropenia developed; R.B.C. elevated; 2 venesections (totaling 1,250 c.c.) made; remission 3 months
8	Z. R.	54, W., F.	7.2	12.9	6,300	1,388,100	2.8 over 4 mo.	4.2	11.0	3,550	339,000	++++	Four months necessary to induce remission which has been satisfactorily held for 14 months and continues
9	B. F.	58, W., M.	6.7	12.8	11,450	349,440	7.0 over 7 mo.	5.54	10.9	9,450	232,680	++	Venesections for 2 years preceding P^{32} therapy; venesections totaling 1,500 c.c. required along with the P^{32} to maintain red cell and hemoglobin equilibrium

*All therapy intravenous unless otherwise indicated.

TABLE II—CONT'D

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY: R.B.C. (PER C.MM.)	ONSET OF THERAPY: HEMOGLOBIN (GM. %)	ONSET OF THERAPY: W.B.C. (PER C.MM.)	ONSET OF THERAPY: PLATELETS (PER C.MM.)	P^{32} DOSAGE* (MC.)	MINIMAL COUNTS UNDER P^{32} THERAPY: R.B.C. (PER C.MM.)	MINIMAL COUNTS UNDER P^{32} THERAPY: HEMOGLOBIN (GM. %)	MINIMAL COUNTS UNDER P^{32} THERAPY: W.B.C. (PER C.MM.)	MINIMAL COUNTS UNDER P^{32} THERAPY: PLATELETS (PER C.MM.)	IMPROVEMENT OF SYMPTOMS	REMARKS
10	H. S.	59, W., M.	(1) 8.9	21.2	17,000	986,000	18 over 6 mo.	4.0	14.8	7,500	297,960	++	Diagnosed 1933 after coronary occlusion; therapy of venesections and roentgen radiation for 8 years with unsatisfactory control
			(2) 7.3	21.4	14,950	648,560	11.0 over 12 mo.	5.85	15.4	9,000	368,000		Moderate resistance to P^{32} therapy; venesections totaling 5,000 c.c. necessary to maintain equilibrium in addition to frequent P^{32} administration
11	E. S.	57, W., F.	6.5	12.9	10,900	5,226,900	8.5 over 6 mo.	3.9	11.5	4,850	213,600	+	Two years of venesections totaled 9,220 c.c. before institution P^{32}—650 c.c. during therapy; note platelet response particularly
12	R. W.	40, W., M.	7.18	15.4	29,000	1,959,000	6.1 over 2½ yr.	7.4	16.6	26,000	1,240,000	+++	Seen at very infrequent intervals; principal complaint was pruritus; small dosage P^{32} would control this symptom satisfactorily for 2- to 4-month periods; hematologic studies too infrequent to evaluate effect on bone marrow; R.B.C. never over 7½ million; a relatively mild trait
13	J. H.	58, W., M.	7.8	11.0	45,000	1,884,000	9.1 over 10 mo.	6.0	8.9	12,500	593,140	+++	Patient expired from carcinoma of bladder; R.B.C., W.B.C., and platelets responded satisfactorily
14	A. K.	33	6.9	11.3	26,500	6,950,000	5.6 over 5 mo.	4.3	9.9	10,100	489,600	++++	P^{32} instituted after development of intolerance to phenylhydrazine therapy after two years. After a 5-month remission had thrombosis of gastric vessels; remission for 1 year

15	K. H.	47, W., F.	(1) 5.7	14.5	14,000	1,495,000	5.4 over 4 mo.	4.09	12.3	6,400	320,000	+++	Remissions lasting (1) 4 months and (2) 12 months induced; as the R.B.C. and hemoglobin levels rise, mental confusion increases
			(2) 6.8	12.8	14,550	480,000	8.1 over 8 mo.	5.2	11.7	5,050	224,120		
16	W. A. R.	60, W., M.	7.9	12.8	24,700	1,249,720	10.6 over 4 mo.	3.6	11.7	7,300	286,560	++++	Venesections and roentgen radiation over spleen for 2 years failed to control adequately R.B.C., W.B.C., or platelet elevations resulting in repeated episodes of thrombosis of arm and leg vessels; remission 1 year with P^{32} therapy (Fig. 3)
17	C. A.	57, W., M.	7.3	21.4	22,800	1,087,800	8.0 over 5 mo.	4.1	13.2	7,700	385,480	++++	Vertigo and early morning headaches markedly relieved; normal hemogram required 5 months of therapy; remission for 9 months continues
18	R. C.	48, W., F.	7.3	13.5	20,000	6,127,000	4.7 over 2 mo.	4.63	12.7	4,950	842,660	+++	In 1942 very small doses of radioactive strontium and phosphorus had no appreciable effect; in 1942-1943 venesections totaling 14,000 c.c. did not maintain the hematologic equilibrium (Fig. 4); normal hemogram in 3 months after therapy began; remission of 20 months; note platelets before and after
19	R. S.	73, W., F.	(1) 7.0	14.2	45,000	4,140,800	16.9 over 5 mo.	3.0	11.0	5,000	522,720	+++	Four years of therapy with venesections failed to control hematologic picture satisfactorily; since 1941 remissions (1) lasting 12 months, (2) 17 months, and (3) 7 months have been induced with series of P^{32} therapy (Fig. 2)
			(2) 7.3	15.0	15,600	1,036,600	7.0	3.6	12.4	3,790	265,300		
			(3) 7.6	18.8	6,200	730,000	9.6 over 10 mo.	5.1	12.1	6,050	466,240		
20	M. D.	65, W., F.	8.5	17.5	20,150	1,260,000	14.0 over 2 mo.	7.0	11.1	9,650	327,600	++++	Four months of excellent clinical improvement continues; hematologic response gradually approaches normal

TABLE III. RADIOACTIVE PHOSPHORUS THERAPY IN CHRONIC LYMPHATIC LEUCEMIA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTIMATED DURATION OF DISEASE BEFORE P^{32} THERAPY	DURATION OF DISEASE AFTER P^{32} THERAPY	P^{32} DOSAGE (MC.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF LYMPHOCYTES	R.B.C. (PER C.MM.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.MM.)				
1	L. R.	57, W., M.	93,000	92	2.2	9.0	150,480	6 mo.	3 wk.	3.5 (o) over 14 days	Developed herpes zoster resistant to roentgen radiation during terminal phase; P^{32} therapy effected symptomatic improvement; no significant hematologic effect
2	T. K.	51, W., M.	132,000	91	2.9	11.3	467,200	1 yr.	4 mo.	9.5 I.V. over 6 wk.	Lymphadenopathy and splenomegaly diminished significantly; W.B.C. 4 mo. later, 9,150 (with 77% lymphocytes); R.B.C., 1,980,000; platelets, 166,000; expired 2 weeks later
3	H. W.	53, W., M.	162,000	89	5.1	12.8	245,760	2½ yr.	1½ yr.	15.8 I.V. over 12 mo. period	Slight diminution in lymphadenopathy; total W.B.C. ranged from 80 to 100,000 while on therapy; R.B.C., 3,300,000 to 4,600,000; hemoglobin, 9.2 to 11.6; pruritis of leucemia cutis adequately controlled
4	G. V.	63, W., M.	3,500	42	3.1	10.2	161,200	2 yr.	7 mo.	4 I.V.	Resistant to roentgen radiation; pruritus of leucemia cutis relieved with P^{32} therapy; lymphadenopathy decreased; no change in hematologic picture except progressive anemia to terminus
5	A. F.	53, W., M.	4,250	50	2.9	8.8	69,840	2 yr.	9 mo.	2.7 I.V. over 4 mo.	Cohnheim type; roentgen radiation fast; no significant hematologic or clinical improvement; progressive downhill course
6	G. W.	65, W., M.	126,000	94	3.48	8.1	313,200	?	1 yr.	3 I.V.	Resistant to roentgen radiation; P^{32} had no significant results

7	B. C.	40, W., M.	136,000	93	3.7	10.7	134,280	?	5 yr.	17 I.V. over 3 mo. period	Received courses of P^{32} therapy in 1940 and 1943,* accomplishing satisfactory remission; January to March, 1945, received 17 mc. P^{32}; lymphadenopathy decreased; June, 1945, W.B.C., 46,000; R.B.C., 2.8; platelets, 5,000; expired Nov. 18, 1945; received only P^{32} over 5-year period
8	C. C.	57, W., M.	82,000	90	3.1	9.2	379,200	?	1 yr.	9 I.V. over 12 mo.	Diagnosis of carcinoma of sigmoid verified by biopsy, coexistent with chronic lymphatic leucemia; the leucemia held under excellent control with P^{32} therapy; patient died of carcinoma with metastases to liver and lung; 1 week before death, W.B.C., 18,000 (62% lymphocytes); R.B.C., 3.0 (Fig. 6)
9	W. S.	45, W., M.	62,000	83	4.7	15.4	399,840	4 yr.	1 yr.	6 over 8 mo.	Received roentgen radiation for 2 years; developed marked hypersensitivity; P^{32} instituted; maintained W.B.C. between 15,000-30,000; platelets fell to 28,000; expired following intracranial hemorrhage, confirmed by post-mortem examination
10	W. C.	55, W., M.	90,000	96	3.3	9.4	93,240	6 mo.	21 mo. (Still under observation)	6 I.V. 4 I.V.	P^{32} continues to control leucemic process very satisfactorily; developed severe hemolytic anemia which was corrected with splenectomy (Fig. 5); therapy continues
11	C. A. S.	77, W., M.	12,700	50	4.8	10.9	492,360	10 mo.	9 mo.	1½ I.V.	The leucemic process under excellent control with roentgen radiation; leucemia cutis developed and pruritus was resistant to roentgen radiation; relieved with P^{32} therapy

*By Dr. John H. Lawrence, University of California.

TABLE IV. RADIOACTIVE PHOSPHORUS THERAPY IN LEUCOSARCOMA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY: W.B.C. (PER C.MM.)	ONSET OF THERAPY: PER CENT OF LYMPHOBLASTS	ONSET OF THERAPY: R.B.C. (MILLIONS PER C.MM.)	ONSET OF THERAPY: HEMOGLOBIN (GM. %)	ONSET OF THERAPY: PLATELETS (PER C.MM.)	ESTIMATED DURATION OF DISEASE BEFORE P^{32} THERAPY	DURATION OF DISEASE AFTER P^{32} THERAPY	P^{32} DOSAGE (MC.)	REMARKS
1	N. B.	3, W., F.	939,000	85	3.2	8.0	259,200	3 wk.	5 wk.	7 over 4 days initially; 6 one mo. later	Reduction in W.B.C., 939,000 to 7,000 with definite improvement clinically; remission lasted 18 days; second elevation of W.B.C. to 485,000 was not effected by second course of P^{32} (Fig. 7)
2	N. M.	5, W., F.	11,800	67	1.5	4.6	21,280	?	3 wk.	2	No change noted
3	M. S.	8, W., F.	72,000	94	21	5.2	16,260	8 wk.	6 wk.	4 over 7 days	600 r. units failed to have any appreciable effect on the blood picture; after 10 days P^{32} instituted; W.B.C. fell from 200,000 to 11,000 in 10 days; terminal roentgen radiation ineffective; expired following massive gastrointestinal hemorrhage
4	M. B.	2, W., F.	102,000	89	2.6	6.5	183,600	6 wk.	3 wk.	2 (o)	Precipitous fall in W.B.C. from 102,000 to 5,500 in 2 days; continued fall to 200 at terminus; marked cervical adenopathy uneffected; 100 r. units to cervical nodes day before death
5	J. W.	7, W., M.	159,000	70	2.9	9.1	167,440	2 mo.	2 mo.	5 over 5 days	Gradual decline of W.B.C. over 2 weeks to 10,350; excellent clinical and hematologic remission for 4 weeks
6	J. D.	8, W., M.	75,000	88	4.5	12.4	336,608	6 mo.	2 mo.	3	Temporary remission of 4 weeks induced; received roentgen radiation during last 2 weeks of life

7	J. M.	5, W., M.	118,000	95	1.6	9.9	9,900	2 mo.	7 days	3	One month previous to P^{32} therapy received course of 650 r. units to various regions showing lymphadenopathy; decline in W.B.C. to 42,000 in 4 days; expired 3 days later; adenopathy slightly less
8	G. S.	3, W., M.	11,600	84	1.8	5.3	48,240	3 wk.		2	No appreciable clinical or hematologic effect during brief observation period; no follow-up
9	M. R.	5, W., M.	2,800	30	1.7	4.4	26,080	6 wk.	1 mo.	2 (o)	Appetite improved; became afebrile for 10 days; no change in adenopathy; no hematologic effect
10	K. C.	5, W., F.	162,000	95	2.06	5.2	32,960	8 mo.	10 days	3	Steady drop in W.B.C. to 900; intracranial hemorrhage terminally
11	C. E.	23, W., M.	61,000	96	3.8	9.9	195,330	3 mo.	3 wk.	3	Gradual fall of W.B.C. to 7,300 in 5 days; rapid re-elevation; expired 3 weeks later
12	B. B.	8, W., F.	200	20	2.1	7.0	100,000	3 wk.	2 days	1½	Staphylococcus septicemia at time of P^{32} therapy; no effect; expired within 48 hours
13	R. W.	4, W., M.	3,000	34	2.06	8.0	16,480	?	8 days	4 during terminal 8 days	W.B.C. fell to 700 day before terminus; R.B.C., to 900,000 with 4 Gm. hemoglobin; clinical course progressively downhill
14	L. H.	5, W., F.	20,000	96	1.7	5.5	10,620	?	6 days	1½	W.B.C. fell to 6,150 day before terminus
15	S. S.	6, W., M.	9,800	68	2.29	8.0	320,600	6 wk.	3 mo.	5	No appreciable benefit clinically or hematologically; received radioactive manganese at another clinic after P^{32} therapy; remission not accomplished
16	M. D.	44, W., F.	36,000	97	2.5	6.2	41,000	11 wk.	1 wk.	10	No clinical change; progressive fall in W.B.C., R.B.C., and platelets
17	P. H.	14, W., F.	169,000	90	3.06	7.9	134,640	6 mo.	7 wk.	8	No clinical change; during last 3 days of life, W.B.C. fell from 131,000 to 2,000
18	A. P.	8, W., M.	434,000	97	2.69	6.0	86,000	5 wk.	5 wk.	6	Transient fall in W.B.C. to 27,000 with corresponding clinical improvement; remission lasted only one week

TABLE V. RADIOACTIVE PHOSPHORUS THERAPY IN MYELOGENOUS LEUCEMIA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTIMATED DURATION OF DISEASE BEFORE P^{32} THERAPY	DURATION OF DISEASE AFTER P^{32} THERAPY	P^{32} DOSAGE (MC.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF MYELOCYTES	R.B.C. (MILLIONS PER C.MM.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.MM.)				
1	J. D.	46, W., M.	193,000	91 (Basophilic myelocytes)	2.9	7.2	123,060	3 wk.	4 days	4.5 (1 dose)	Diagnosis: Acute basophilic leucemia; no improvement clinically or hematologically; expired 4 days after therapy; postmortem examination confirmed clinical impression
2	E. S.	24, W., M.	474,000	95	2.34	4.7	56,160	2½ yr.	26 days	5 (1 dose)	Received intensive roentgen radiation during preceding 2 years which had failed to control the peripheral blood picture or the marked splenomegaly adequately; progressive increase in W. B.C. to terminus; P^{32} had no effect on spleen size
3	W. C. W.	35, W., M.	173,000	75	3.5	12.8	653,380	6 mo.	2 yr.	26.6 over 1-year period	Peripheral W.B.C. maintained between 3 to 5,000 and R.B.C. 3.5 to 5.0 satisfactorily; spleen failed to diminish in size adequately, therefore roentgen radiation totaling 800 r. given over the spleen; due to limited supply P^{32}, roentgen radiation used entirely for last year of life
4	E. C.	51, W., M.	70,000	38	3.7	11.4	1,159,740	12 mo.	5 mo.	19 over 5 months	After three months of P^{32} therapy there was a flare-up of an old osteomyelitis of mandible; 400 r. units given over adjacent moderate lymphadenopathy; W.B.C. ranged 8 to 20,000; R.B.C., 3.3 to 4.5; hemoglobin 9.2 to 12.5 Gm.; platelets fell to 33,000 terminally; splenomegaly adequately controlled

5	J. J.	36, W., M.	189,000	38	2.9	9.1	560,240	15 mo.	5 mo.	Radioactive strontium, 1.1 over 3 weeks; P^{32} 11.5 over 4 weeks	Neither radioactive strontium nor phosphorus had appreciable clinical or hematologic response; roentgen radiation to spleen brought about temporary remission; expired 6 months after onset of therapy
6	R. M.	61, W., F.	30,300	25	3.58	7.0	436,760	12 mo.	26 mo.	24 over 10 months	P^{32} therapy maintained a mean W.B.C. of approximately 16,000; R.B.C., 3.8; hemoglobin, 10.5 Gm. for 18 months; splenomegally was principal source of symptoms; roentgen radiation therapy for last 10 months of life
7	C. R.	51, W., F.	54,000	43	3.9	10.2	555,220	16 mo.	10 mo.	38 (o) 7 months	Developed hypersensitivity to roentgen radiation after 16 months; W.B.C. not adequately controlled (mean W. B. C. 95,000); R.B.C., 3.5 to 4.6; hemoglobin, 9.8 to 12.3 Gm.; splenomegaly controlled; remission 10 months
8	I. E. T.	33, W., M.	125,000	47	4.4	13.0	674,000	3 yr.	10 mo.	$8\frac{1}{2}$ over $2\frac{1}{2}$ months	No previous therapy; excellent remission with control of splenomegaly 1 year; W.B.C. 22 to 54,000; R.B.C., 4.2 to 4.6; hemoglobin, 12.5 to 13 Gm.; now receiving second course of therapy
9	G. M.	35, W., M.	294,000	54	3.3	6.9	893,700	$1\frac{1}{2}$ yr.	7 mo.	10 over 7 months	Unable effectively to lower W.B.C. below 100,000; no significant effect on splenomegaly; R.B.C., 3 to 4.2; hemoglobin, 6.0 to 10.5 Gm.
10	A. W.	62, W., F.	103,000	31	3.6	10.5	796,400	3 yr.	1 yr.	17 over 1 year	Developed severe reactions to roentgen radiations after $2\frac{1}{2}$ years; Fowler's solution (4 months) ineffective; P^{32} held satisfactory hematologic remission; W.B.C., 8 to 50,000; R.B.C., 3.2 to 3.9; hemoglobin 8 to 11.4 Gm.; no effect on marked splenomegaly (Fig. 8)

TABLE V—CONT'D

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTIMATED DURATION OF DISEASE BEFORE P^{32} THERAPY	DURATION OF DISEASE AFTER P^{32} THERAPY	P^{32} DOSAGE (MC.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF MYELOCYTES	R.B.C. (MILLIONS PER C.MM.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.MM.)				
11	M. L.	55, W., F.	297,000	63	2.9	7.7	623,280	3 yr.	Continues 1 yr.	12 over 4 weeks	Reduced W.B.C. to 118,000 in 3 weeks; elevation of R.B.C. from 2.9 to 3.8; hemoglobin, 7.7 to 10.1 Gm.; clinically little change; roentgen radiation instituted
12	J. B.	41, W., M.	261,000	78	3.9	9.5	435,500	2 yr.	22 mo.	14 over 22 months	P^{32} has maintained W.B.C. mean of 75,000 over 22-month period; R.B.C., 4.5, and hemoglobin, 12 Gm., without transfusions; splenomegaly controlled satisfactorily
13	R. M.	49, W., M.	100,000	42	3.0	8.9	1,043,280	3 yr.	24 mo.	37 over 22 months	Developed marked sensitivity to roentgen radiation after 3 years of successful control; P^{32} therapy for 22 months has not satisfactorily controlled W.B.C. (averaged 175,000) and splenomegaly; R.B.C. mean 3.0, with 9.1 Gm. of hemoglobin
14	B. R.	52, W., F.	176,000	43	3.7	7.5	677,040	2 yr.	9 mo.	13 over 3 months	Developed marked intolerance to both roentgen and urethane therapy; P^{32} gradually reduced W.B.C. with corresponding rise in R.B.C., but splenomegaly not appreciably effected

TABLE VI. RADIOACTIVE PHOSPHORUS THERAPY IN MONOCYTIC LEUCEMIA

TABLE VI. RADIOACTIVE PHOSPHORUS THERAPY IN MONOCYTIC LEUCEMIA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					DURATION OF DISEASE AFTER INSTITUTION OF P^{32} THERAPY	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF MONOCYTES	R.B.C. (MILLIONS PER C.MM.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.MM.)		
1	D. M.	68, W. F.	26,800	89 (monocytes)	3.65	6.1		21 mo.	A total of 25 mc. (o) over 21-month period effected a moderate improvement clinically; the spleen diminished in size during the first 12 months of therapy; the total W.B.C. was not appreciably altered; there was a slow and progressive rise to a terminal W.B.C. of 284,000 with 97% monoblasts
2	J. F. B.	3, W., M.	39,550	67 (monoblasts)	4.16	11.9	174,720	6 wk.	2 mc. P^{32} orally was followed by a fall in the W.B.C. from 39,550 to 6,100 in 7 days
3	C. H.	5, W., F.	526,000	96 (monoblasts)	2.7	6.3	137,160	7 wk.	5 mc. P^{32} followed by fall in W.B.C. to 8,600 in 21 days; second injection of 1½ mc. had no appreciable effect on the blood picture
4	A. K.	45, W., F.	3,950	45 (monoblasts)	2.8	10.5	40,460	4 mo.	25 mc. P^{32} orally over 4-month period failed to achieve any beneficial effect; there was a steady rise to a terminal W.B.C. of 171,000
5	E. G.	64, W., F.	30,300	70 (monoblasts)	3.03	11.2	133,320	4 wk.	2 mc. P^{32} (I.V.) had no effect over a 4-week observation period
6	E. S.	54, W., M.	165,000	81 (monoblasts)	2.5	8.5	15,180	4 days	0.1 mc. radioactive strontium (o) ineffective; steady rise in W.B.C. followed by 4.2 mc. P^{32}; continued rise in W.B.C. to 308,000 and terminus 4 days later
7	R. C.	43, W., F.	34,900	12 (monoblasts)	2.97	10.2	118,800	4 days	4.8 mc. P^{32} (I.V.) over 2-day period effected no results; steady rise in W.B.C. to 69,000 4 days later
8	A. W.	59, W., M.	127,000	95 (monoblasts)	2.1	7.4	42,800	4 wk.	5 mc. P^{32} (I.V.); no appreciable change in hematologic picture except progressive anemia and thrombocytopenia; no effect on W.B.C.
9	J. W.	32, W., M.	253,000	81 (monoblasts)	3.9	10.5	132,000	12 wk.	6 mc. (over 3 days); lymphadenopathy diminished; fall in W.B.C. from 253,000 to 88,000 in 14 days; W.B.C. 2½ months after therapy 4,100 (Fig. 10)
10	P. C.	38, W., M.	16,650	79 (monoblasts)	3.13	8.3	87,000	9 wk.	Received 5 mc. P^{32} over six weeks; no significant clinical or hematologic change

TABLE VII. RADIOACTIVE PHOSPHORUS THERAPY IN MISCELLANEOUS DISEASES

CASE	PATIENT	AGE, RACE, AND SEX	DIAGNOSIS	P^{32} DOSAGE (MC.)	SUBJECTIVE RESULTS	OBJECTIVE RESULTS	HEMATOLOGIC RESULTS
1	C. M. C.	58, W., M.	Carcinoma of prostate (biopsy) with metastasis to cranium, pelvis, lumbar spine,	11 over 3-month period	Excellent transient relief of bone pain	No change roentgenologically	R.B.C. fell from 3.5 to 1.4 million and W.B.C., from 8,600 to 1,850 after 2 months of therapy
2	A. H.	25, W., M.	Astrocytoma of cerebellum with metastasis to spleen, bone marrow	17 over 3½-month period	Temporary relief of bone pain	None	Moderate leucopenia (2,600) developed during therapy
3	J. H.	19, W., M.	Retroperitoneal sarcoma with pulmonary metastasis	5	None	None	None
4	E. N.	68, W., M.	Exfoliative dermatitis	7½ over 4-month period	Pruritus markedly relieved	Dermatitis improved significantly	Moderate diminution of platelets, R.B.C., and granulocytes during therapy (Fig. 1)
5	S. F.	54, W., F.	Multiple myeloma	3 (patient was in terminal state)	None	None	None
6	J. W.	37, W., M.	Multiple myeloma	11.5 over 7-month period	Bone pain satisfactorily controlled for 7 months; roentgen radiation used terminally due to inadequate supply P^{32}	None	None
7	S. A. L.	48, W., M.	Multiple myeloma	15 (o) over 6-week period	Deep bone pain partially relieved for 7 to 10 days following each administration	None	None
8	L. L. V.	43, W., M.	Mycosis fungoides	5.3 (o) over 6-week period	None—progressive downhill course to terminus 2 months after onset of therapy	None	None
9	E. D.	50, W., F.	Carcinoma of breast with metastasis to vertebrae, ribs, liver, and lungs	5.0	No significant relief from bone pain	None	None
10	C. J.	35, W., M.	Seminoma of testicle with generalized metastasis	5.0	No relief from bone pain	None—no rotentgenologic evidence of regression of bone lesions: no regression of subcutaneous nodules	None
11	E. M.	48, W., M.	Multiple myeloma	9.5 over 3 months	Clinical status stationary for 6 months	No change roentgenologically	Transient leucopenia (3,350 per c.mm.) developed during therapy

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FOLIC ACID IN THE MAINTENANCE OF PERNICIOUS ANEMIA

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THE first report on the effect of folic (pteroylglutamic) acid in the treatment of macrocytic anemia appeared in November, 1945.[1] Since then numerous reports describing the results obtained in the treatment of pernicious anemia,[2] macrocytic anemia of nutritional deficiency,[3] sprue,[4] macrocytic anemia of pregnancy,[2] and macrocytic anemia of infancy[5] have appeared. In general, folic acid has been found to cause a hematologic improvement similar to or identical with that of liver extracts when given to patients with macrocytic anemia in relapse, as judged by the response of the reticulocytes, erythrocytes, white cells, and platelets. In sprue, in addition, there is a rapid subsidence of diarrhea and a marked improvement in intestinal absorption. One author, however, after study of a series of cases of pernicious anemia,[6] has concluded that folic acid does not produce as great a reticulocyte response or as complete return to normal of erythrocytes as occurs with liver extracts. Further, he concluded that a combination of liver extract and folic acid is more effective than either alone. These latter conclusions were based upon the observation that reticulocyte response to the combined therapy was greater than might have been expected in some of the cases and the increase in erythrocytes was somewhat more rapid than occurred with either alone. This study compared results obtained in different patients, rather than in the same patient, using as the basis for comparison successive periods of treatment with folic acid, with liver extract, and with a combination of the two. Because of the well-known variation of response in different patients, the validity of comparisons made in different individuals is doubtful, unless the number of comparisons made was sufficient to permit statistical evaluation.

Neurologic relapse has been observed during therapy with folic acid in patients with pernicious anemia, even when the blood was well maintained at normal levels and in the absence of macrocytosis.[6-8]

The study reported here was undertaken to determine the effectiveness of synthetic folic acid (pteroylglutamic acid)* in maintenance therapy of pernicious and allied macrocytic anemia, and an attempt has been made to compare such treatment with liver extract therapy.

I. MATERIAL AND METHODS

The study included 41 patients with proved macrocytic anemia. In 35, the results of folic acid therapy were compared with previous antipernicious anemia therapy with intra-

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muscular injections of crude and purified liver extracts, oral liver-stomach therapy (Extralin, Lilly) and stomach therapy (Ventriculin, Parke, Davis & Co.). Folic acid therapy was given to 6 patients who were in severe hematologic relapse.

The folic acid was administered parenterally to 25 patients and orally to 16 patients. The intramuscular route was used for all parenteral injections, except in one patient, who insisted upon intravenous administration. The parenteral dose was 50 mg. in 15 patients and 150 mg. in 9 patients. Intervals between injections of from two to six weeks were employed, although the usual interval was either three or four weeks. The duration of this interval was determined, whenever possible, by the previous intervals between injections of liver extract.

When given orally, folic acid was administered in daily doses of 10 to 40 mg.; the usual dose was either 10 or 20 milligrams. No patient in this series was treated with a daily oral dose of less than 10 milligrams.

There were 22 women and 19 men in the series; of these, 33 were white and 8 were Negro. The age of the patients ranged from 37 to 82 years, the average age being 62.4 years. Three patients developed macrocytic anemia following gastric resection and one patient had macrocytic anemia in association with syphilitic fibrosis of the stomach. The others were well-substantiated cases of addisonian pernicious anemia, as evidenced by the presence of macrocytic anemia, histamine-fast achlorhydria, response to liver extracts or stomach concentrate and, in some cases, the presence of combined system disease and megaloblastic hyperplasia of the bone marrow.

The incidence of degenerative diseases was high, as would be expected in a group of patients in this age range. In addition, 5 patients had syphilis and 2 had diabetes; 3 had had gastric resection, 2 for peptic ulcer and 1 for carcinoma of the stomach.

Because of the errors inherent in the determination of the erythrocyte counts and because the determinations made before the institution of folic acid therapy were performed by different technicians, it was arbitrarily decided that differences in the erythrocyte count of less than 300,000 per cubic millimeter would not be considered significant. A reduction of 300,000 to 500,000 cells per cubic millimeter was considered a moderate decrease; that of 500,000 per cubic millimeter or more was considered a marked decrease. Conversely, an increase of 300,000 cells per cubic millimeter or more was considered evidence of improvement.

In Tables I to IV the values for the erythrocyte count and hemoglobin level prior to folic acid therapy represent the average of many determinations over a long period of time. Values obtained at the end of the period of observation on folic acid therapy represent an average of at least three determinations. Hematocrit values were not regularly determined, either before or after the institution of folic acid therapy. The authors are cognizant of the fact that without hematocrit determinations definite conclusions concerning macrocytosis cannot be drawn.

All the patients were carefully examined, before and during the course of therapy with folic acid, for changes in deep tendon reflexes, diminution of vibratory sensation, astereognosis, muscle coordination, and the presence of positive Babinski and Romberg signs. An attempt was made to evaluate the nutritional status, as judged by weight and evidence of vitamin deficiency (glossitis, cheilosis, stomatitis, gingivitis). The patients were carefully and repeatedly questioned regarding subjective changes in their state of well-being.

II. RESULTS

A. Hematologic Observations.—The patients were divided into two groups: (1) those studied six to twelve months and (2) those studied less than six months.

The first group, consisting of 32 patients, was studied six to twelve months, with an average of 10.7 months. Of these, 23 or 71.9 per cent, were maintained in a satisfactory hematologic state. Eighteen of the 23 (Table I) showed no

TABLE I. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED NO SIGNIFICANT CHANGE IN ERYTHROCYTE LEVELS

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTERVAL BETWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A. R.B.C. ×10⁶	AV. BLOOD VALUES FOR YEAR BEFORE F.A. HB. (GM.)	AV. BLOOD VALUES AFTER F.A. R.B.C. ×10⁶	AV. BLOOD VALUES AFTER F.A. HB. (GM.)	CHANGE R.B.C. ×10⁶	CHANGE HB. (GM.)
Group I—Treated for Six to Twelve Months													
1	82	M	W	12	150	3 wk.	I.V.	3.60	12.7	3.41	11.6	−0.19	−1.1
2	52	F	W	12	150	3 wk.	I.M.	4.65	13.6	4.38	13.1	−0.27	−0.5
3	67	M	W	10	150	3 wk.	I.M.	4.32	13.9	4.51	13.6	+0.19	−0.3
4	50	F	W	11	150	4 wk.	I.M.	4.51	13.9	4.39	13.4	−0.12	−0.5
5	72	F	W	11	150	4 wk.	I.M.	4.38	14.0	4.12	13.8	−0.26	−0.2
6	48	F	W	11	50	2 wk.	I.M.	4.03	12.5	3.99	12.3	−0.40	−0.2
7	53	F	W	11	40	3 wk.	I.M.	4.65	13.9	4.40	13.8	−0.25	−0.1
8	63	F	W	11	50	3 wk.	I.M.	4.63	13.4	4.44	13.6	−0.18	+0.2
9	81	M	W	12	50	4 wk.	I.M.	4.48	13.4	4.69	13.4	+0.21	0
10	57	F	W	12	50	4 wk.	I.M.	4.20	13.9	4.45	13.8	+0.25	−0.1
11	56	M	W	7	50	4 wk.	I.M.	4.33	14.9	4.14	13.9	−0.19	−1.0
12	67	M	W	10	20	Daily	Oral	4.25	13.6	4.31	12.9	+0.60	−0.7
13	64	F	W	12	10	Daily	Oral	4.09	12.8	3.97	12.3	−0.12	−0.5
14	73	M	W	12	10	Daily	Oral	4.43	14.4	4.26	14.4	−0.17	0
15	57	F	W	12	10	Daily	Oral	4.44	12.9	4.73	14.0	+0.29	+1.1
16	65	F	W	7	10	Daily	Oral	4.56	14.0	4.85	14.7	+0.29	+0.7
17	78	F	W	7	10	Daily	Oral	4.52	13.1	4.45	13.3	−0.07	+0.2
18	74	M	W	6	10	Daily	Oral	4.48	13.8	4.51	13.9	+0.03	+0.1
Group II—Treated Less Than Six Months													
19	59	F	W	3	50	4 wk.	I.M.	4.55	13.4	4.54	13.4	−0.01	0
20	46	F	C	4	10	Daily	Oral	4.48	12.5	4.26	10.2	−0.22	−2.3

significant change in erythrocyte count or in hemoglobin level, in comparison with previous findings obtained following therapy with liver extract; in three cases, improvement was manifested (Table III). One patient (Case 31, Table III) was not in good hematologic remission before institution of folic acid therapy. She was a 51-year-old white woman who was extremely sensitive to substances in liver extract and who had been maintained on nine to fifteen capsules daily of an oral liver-stomach preparation, Extralin B (Lilly), on which therapy normal blood values were not obtained. Another patient (Case 32, Table III) had also been maintained on Extralin B, because of marked sensitivity to intramuscular injections of liver extract, and her erythrocyte count and hemoglobin level were well below normal when folic acid therapy was instituted. Two patients in this group were started on folic acid therapy when in relapse; they had received no liver extract therapy for several months prior to this time (Table IV). These patients showed satisfactory clinical and hematologic improvement.

In 2 of this group of 32 patients a moderate decrease in erythrocyte count occurred, while in 7 a marked decrease developed, as already defined (Table II).

The second group consisted of nine patients studied for one to five months (average, 3.3 months). None of these showed any significant decrease in erythrocyte count. Two showed no appreciable change (Table I) and 3 showed an increase in erythrocyte count, as compared with previous liver extract therapy (Table III). The other four were started on folic acid therapy when in re-

TABLE II. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED MODERATE (300,000 TO 500,000 PER CUBIC MILLIMETER) OR MARKED (OVER 500,000 PER CUBIC MILLIMETER) DECREASE IN ERYTHROCYTE LEVELS

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTERVAL BETWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A. R.B.C. ×10⁶	AV. BLOOD VALUES FOR YEAR BEFORE F.A. HB. (GM.)	AV. BLOOD VALUES AFTER F.A. R.B.C. ×10⁶	AV. BLOOD VALUES AFTER F.A. HB. (GM.)	CHANGE R.B.C. ×10⁶	CHANGE HB. ('NO)
Group I—Treated for Six to Twelve Months Moderate Decrease in Erythrocyte Count (300,000 to 500,000 Per Cubic Millimeter)													
21	51	F	C	11	150	4 wk.	I.M.	4.53	14.0	4.11	13.4	−0.42	−0.6
22	58	M	C	11	50	4 wk.	I.M.	4.19	12.9	3.85	12.7	−0.34	−0.2
Group I—Treated for Six to Twelve Months Marked Decrease in Erythrocyte Count (Over 500,000 Per Cubic Millimeter)													
23	59	M	W	11	150	4 wk.	I.M.	3.76	13.4	2.55	9.8	−1.21	−3.6
24	75	M	W	12	150	6 wk.	I.M.	4.15	13.9	3.56	12.5	−0.59	−1.4
25	70	F	W	9	150	6 wk.	I.M.	4.48	13.8	3.75	12.8	−0.73	−1.0
26	61	F	W	12	50	3 wk.	I.M.	4.73	13.8	3.43	10.8	−1.30	−3.0
27	80	F	W	12	50	3 wk.	I.M.	4.19	13.4	3.48	12.2	−0.71	−1.2
28	37	M	C	12	50	4 wk.	I.M.	4.34	14.2	3.39	13.6	−0.95	−0.6
29	57	M	C	8	50	4 wk.	I.M.	4.79	13.3	4.11	11.3	−0.68	−2.0

TABLE III. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED AN IMPROVEMENT IN ERYTHROCYTE LEVELS (MORE THAN 300,000 PER CUBIC MILLIMETER)

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTERVAL BETWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A. R.B.C. ×10⁶	AV. BLOOD VALUES FOR YEAR BEFORE F.A. HB. (GM.)	AV. BLOOD VALUES AFTER F.A. R.B.C. ×10⁶	AV. BLOOD VALUES AFTER F.A. HB. (GM.)	CHANGE R.B.C. ×10⁶	CHANGE HB. (GM.)
Group I—Treated for Six to Twelve Months													
30	76	F	W	11	50	4 wk.	I.M.	4.76	14.9	5.07	15.1	+0.31	+0.2
31	51	F	W	12	10 to 40	Daily	Oral	3.71	12.2	4.04	12.7	+0.33	+0.5
32	77	F	W	0	20	Daily	Oral	3.04	10.6	4.16	11.4	+1.12	+0.8
Group II—Treated for Less Than Six Months													
33	69	F	W	5	50	2 wk.	I.M.	4.48	13.6	4.92	13.4	+0.44	−0.2
34	76	F	W	1	45	2 wk.	I.M.	4.15	12.8	4.59	13.1	+0.44	+0.3
35	78	M	W	3	20	Daily	Oral	4.15	12.9	4.71	12.8	+0.56	−0.1

lapse without having had recent previous liver extract therapy (Table IV). A satisfactory hematologic remission ensued in every case. One patient (Case 40, Table IV), at the beginning of treatment, had an erythrocyte count of 340,000 per cubic millimeter; hemoglobin, 1.4 Gm. per 100 ml. of blood; leucocyte count, 1,200 per cubic millimeter; hematocrit, 4.0; mean corpuscular volume, 117.5 cubic microns; mean corpuscular hemoglobin, 41.2 micromicrograms; mean corpuscular hemoglobin concentration, 35 per cent. These values are the average of determination done in triplicate. In our experience they represent the most severe anemia ever seen in pernicious anemia from which the patient recovered.

It should be noted that in the entire series of 41 patients, only 9 had a significant decrease in the erythrocyte count as compared with previous liver

TABLE IV. PATIENTS TREATED WITH FOLIC ACID FROM RELAPSE

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTERVAL BETWEEN DOSES	ROUTE	BLOOD AT START OF F.A. THERAPY		AV. BLOOD VALUES AFTER F.A.		CHANGE	
								R.B.C. $\times 10^6$	HB. (GM.)	R.B.C. $\times 10^6$	HB. (GM.)	R.B.C. $\times 10^6$	HB. (GM.)
Group I—Treated for Six to Twelve Months													
36	47	F	C	11	20	Daily	Oral	1.00	3.9	3.90	10.9	+2.90	+7.0
37	76	F	W	11	20	Daily	Oral	3.10	11.9	4.24	13.6	+1.14	+1.7
Group II—Treated for Less Than Six Months													
38	52	M	C	5	50	4 wk.	I.M.	1.02	3.4	4.58	13.6	+3.56	+10.2
39	49	F	W	5	30 to 40	Daily	Oral	2.42	11.4	4.75	13.9	+2.33	+2.5
40	45	M	C	4	10	Daily	Oral	0.34	1.4	4.81	11.5	+4.47	+10.1
41	68	M	W	2	10	Daily	Oral	1.80	5.6	5.09	15.6	+3.29	+10.0

extract therapy (Table II). But, in all these cases, injections were at intervals of three to six weeks; in 5 patients the individual dose was 50 mg., while in the other 4 the dose given was 150 milligrams. The size of the individual dose was not correlated with the severity of the relapse in this group. None of the patients receiving oral therapy had hematologic relapse.

On the other hand, 6 patients of the entire series of 41 showed definite improvement in erythrocyte values as compared with previous liver extract therapy (Table III). Of these, 3 were receiving daily oral medication, 2 were receiving either 45 or 50 mg. at intervals of two weeks, and only 1 was given 50 mg. at intervals greater than two weeks. It should be noted that with the exception of the last patient, those showing improvement were either on daily oral therapy or were receiving intramuscular injection at intervals of two weeks, the shortest period used in this study.

Of the 6 patients showing improvement, 2 (Cases 31 and 32, Table III) had blood levels definitely below normal values at the time of institution of folic acid therapy, while 2 others (Cases 34 and 35, Table III) had blood levels suggestively lower than normal. The improvement obtained with folic acid therapy, therefore, probably indicates that the patients had not been receiving enough liver extracts previously or, as in the case of the patients (Cases 31 and 32), who had been on oral liver extract therapy, were not absorbing the liver extract efficiently enough to maintain normal blood levels.

Of the patients showing hematologic relapse, one case is of particular interest. He was a 59-year-old Italian who had a diagnosis of pernicious anemia established many years before and who had been treated regularly with intramuscular liver extract injections. Recent liver extract therapy had not maintained the blood at normal levels, possibly because of insufficient dosage at insufficiently frequent intervals. When transferred to parenteral folic acid therapy (150 mg. at four-week intervals) there was a further marked drop in erythrocytes and hemoglobin, although the patient stated that he felt well (Fig. 1). He was admitted to the hospital for study, and a reticulocyte count made on the fifth day after the last dose of folic acid revealed a value of 13 per cent

(Fig. 1). Without further treatment the reticulocytosis subsided and there was an increase in red count and hemoglobin. At the end of four weeks, however, the erythrocytes and hemoglobin had again decreased. The rapidity of decrease in the erythrocyte count suggested the presence of a hemolytic process. Determinations of icterus index, serum bilirubin, van den Bergh, and quantitative urine urobilinogen did not support this idea, however. Studies of fecal urobilinogen were not made. It seems likely that each injection of folic

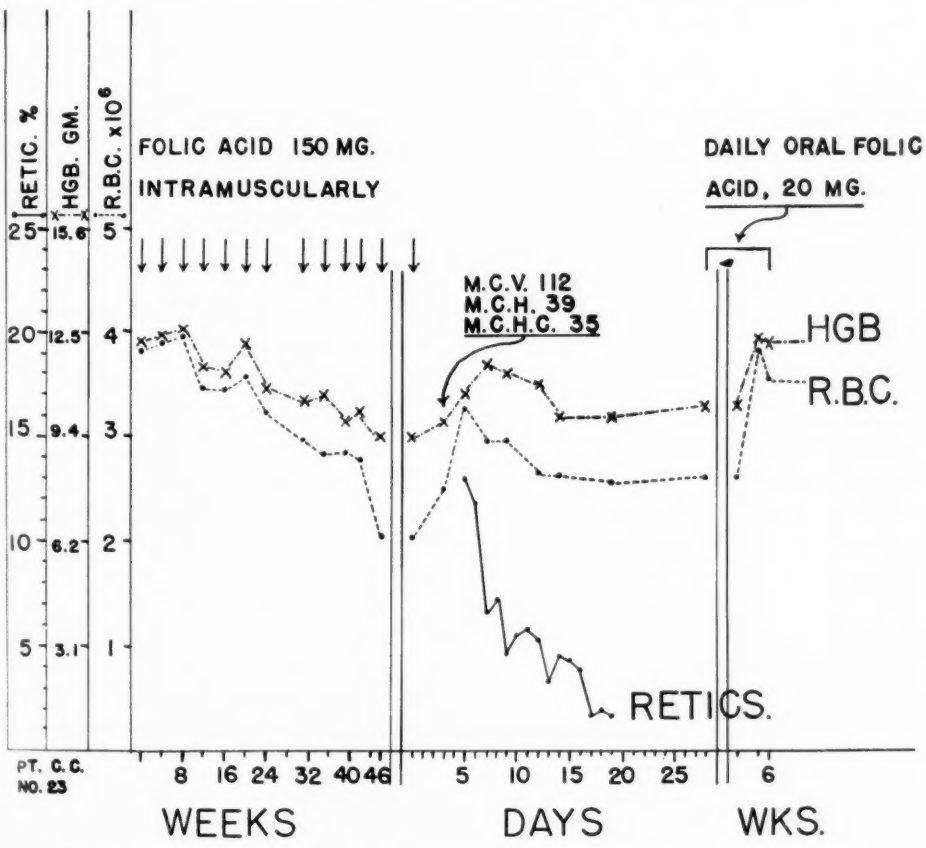

Fig. 1.—Patient (Case 23, Table II) showing hematologic relapse on folic acid therapy, temporary improvement after single injection of folic acid, and improvement after therapy with daily oral doses of folic acid.

acid in this patient caused a characteristic reticulocyte response with increase in erythrocytes and hemoglobin, but this response had ended and the patient had relapsed a little further by the end of each four-week interval. Thus, the course was probably one of alternate responses and relapses, with each relapse carrying the blood values a little lower than before. Such a finding is not surprising in view of our knowledge of folic acid excretion. Steinkamp and co-workers[9] have shown that a large part of administered folic acid is excreted in a relatively short time. Welch and Heinle[10] have been able to demonstrate little, if any, "storage" of folic acid from study of urinary excretion.

When the patient was placed on oral therapy with folic acid, 10 mg. daily (Fig. 1), there was an improvement in the erythrocyte count to a level maintained when the patient was receiving liver extract. Folic acid, however, like the previous liver extract therapy, was unable to produce a normal erythrocyte count in this patient. Bone marrow studies, including fixed sections of particles obtained by sternal aspiration, did not reveal an unusual amount of fat in the marrow.

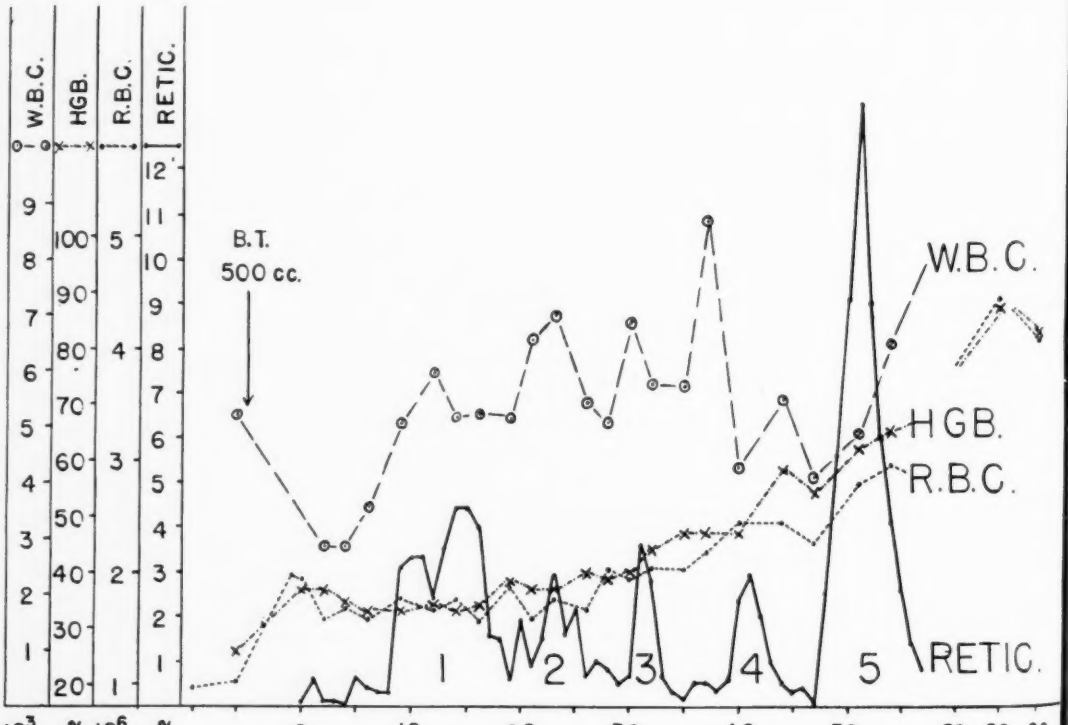

Fig. 2.—Patient L. P. 100% hemoglobin=15.6 Gm. per 100 milliliter. *Xanth.*=xanthopterin. *L.E.*=Lederle solution of liver extract, 15 units per milliliter. *B.T.*=Blood transfusion.

Outright failure of response to folic acid has not been reported. One patient observed by us showed very little response to doses of folic acid which have provided a good, if not theoretically maximal, response in all our other patients. This patient was a 46-year-old colored woman with a macrocytic anemia, histamine-fast achlorhydria, and megaloblastic hyperplasia of the bone marrow. During a period of ten days (Fig. 2) she was given daily intramuscular injections of 1 mg. of folic acid with a delayed and very small reticulocyte response (*1*, Fig. 2). During a second period of eleven days the daily intra-

muscular dose of folic acid was increased to 6 mg. with a second, delayed, very small reticulocyte response (*2*, Fig. 2). During a third period of twelve days, the daily intramuscular dose of folic acid was increased to 12 mg. with another very small reticulocyte response (*3*, Fig. 2). During a fourth period of thirteen days the daily intramuscular injections of 12 mg. of folic acid were continued with the addition of daily oral doses of 36 mg. of xanthopterin. A fourth small reticulocyte response (*4*, Fig. 2) followed. During this entire period of forty-six days there was only a very slight increase in erythrocytes and hemoglobin. She was then given daily intramuscular injections of 15 units of purified liver extract (Lederle, 15 units per milliliter), and on the fifth day after the start of this therapy a theoretically maximal reticulocyte response occurred (*5*, Fig. 2) and there was a rapid increase in erythrocytes and hemoglobin. The folic acid used in treating this patient was assayed with *Lactobacillus casei* and found to be as potent as labeled. Unfortunately, it was not possible to carry out studies of urinary excretion in this patient.

The reason for failure of response to a dose of folic acid sufficient to produce a good response in all of our other patients is not clear nor is there an obvious explanation for the prompt response to liver extract after failure of folic acid. We have not encountered any other such patients to date, but a similar failure in two patients of Shukers has been referred to by Darby.[11]

The patient was continued on liver extract therapy for ten months, at the end of which time the erythrocyte count was 4,510,000 per cubic millimeter and the hemoglobin level was 12.6 Gm. per 100 milliliters. At that time she was transferred to folic acid therapy and given daily oral doses of 10 milligrams. During a period of five months on folic acid therapy there has been no significant change in blood values, body weight, or symptoms.

B. General Clinical Observations.—The majority of the patients noted no difference in subjective sense of well-being. The few who did not feel as well were among those receiving intermittent intramuscular injections with decrease in blood values. These patients observed that they felt well for a short time after each injection of folic acid but that the effect did not last as long as with liver extract injections. There were a few patients, particularly those receiving oral medication, who claimed subjective improvement, especially an increase in appetite, after institution of folic acid therapy. No evidence of associated vitamin deficiencies was observed in any of these patients. Body weight was maintained in all cases except in those who showed obvious hematologic relapse.

C. Neurologic Status of Patients.—Neurologic relapse occurred in three of these forty-one patients. The most striking of these has been reported in detail in a previous publication.[7] The patient, who had addisonian pernicious anemia, was seen in relapse (Case 41, Table IV). Folic acid therapy was begun in daily oral dosage of 10 milligrams. This was followed by a reticulocyte response of 40 per cent (calculated theoretic maximum, 22.3 per cent). There was an excellent rise in erythrocytes and hemoglobin, with complete disappearance of macrocytosis. Eighty-three days after beginning treatment there was a sudden and rapidly progressive onset of paresthesias with rapid loss of deep reflexes and vibratory sensation. At the time of onset of the neurologic relapse, the

blood findings were: erythrocytes, 5,090,000 per cubic millimeter; hemoglobin, 15.9 Gm. per 100 ml.; hematocrit, 48; mean corpuscular volume, 94.5 cubic microns. Vigorous liver extract therapy for twenty-seven days was necessary to produce the first evidence of arrest of the neurologic lesions. Since then the patient has made gradual and steady improvement until there is only a small amount of residual numbness of the fingers and toes. Deep reflexes are still absent and vibratory sensation has not improved. This is in keeping with the concept that degenerative lesions in the spinal cord are not reversible,[12] whereas paresthesias and peripheral neuritis are responsive to therapy.

This patient ate an inadequate diet throughout. At the time of institution of therapy he had only four teeth and this, combined with the naturally poor appetite seen in patients with untreated pernicious anemia, reduced his food intake. Shortly after remission was induced with folic acid he had the four remaining teeth extracted and the resulting sore mouth and complete absence of teeth limited his food intake to liquids for about three weeks. It should be emphasized that after hematologic remission was induced with folic acid the patient had a desire to eat but was restricted from doing so by the absence of teeth.

Another patient (Case 31, Table III) was a 51-year-old white woman with addisonian pernicious anemia who had developed severe sensitivity to substances in liver extract. After an unsuccessful trial of desensitization, she was placed on oral therapy with Extralin B (Lilly), 9 to 15 capsules daily. This dosage did not maintain the blood at satisfactory levels, and liver extract sensitivity was manifested by a continuous mild skin eruption. She was placed on oral folic acid therapy of 40 mg. daily, reduced gradually to 10 mg. daily. There was a marked improvement in the blood with an associated improvement in sense of well-being. The skin eruption completely disappeared. After a year, however, she developed soreness of the tongue and mouth, without objective changes, and increasing numbness of the feet and hands, the latter especially noticeable since the patient was a seamstress. Therapy with Extralin B was reinstituted at the rate of 6 capsules daily, and folic acid, 40 mg. daily, was given simultaneously. Over a period of weeks the soreness of the mouth disappeared and the numbness of the extremities improved. The skin eruption promptly returned. The patient also probably had inadequate food intake. Her appetite was never robust. She was an extremely "nervous" individual and when emotionally disturbed, which was frequently, she responded with nausea and vomiting so that she would eat little or nothing for two or three days at a time. Repeated x-ray examinations failed to reveal any organic disease of the gastrointestinal tract.

The third patient showing neurologic relapse was a 77-year-old white woman who had been maintained on weekly or biweekly injections of liver extract for many years with satisfactory blood values (Case 34, Table III). This patient also had a poor appetite, with considerable "indigestion," so that she voluntarily limited food intake with respect to both quantity and quality. She was changed to folic acid therapy, 45 mg. intramuscularly every two weeks. After only one month she developed slight but definite increase in numbness of the hands, soreness of the tongue, weight loss, and further decrease in appetite and

increase in "indigestion." She was again placed on intramuscular liver extract therapy (purified liver extract, 15 units per milliliter, Lilly) at weekly intervals and was given a hydrochloric acid-yielding tablet (Acidulin, Lilly) with meals. In a period of days the soreness of the tongue and numbness of the extremities improved and eventually disappeared. Her appetite increased to a degree which astonished her family and she became free of indigestion. Some credit for this improvement, at least as concerns the indigestion, should probably be given to the hydrochloric acid since she is in a better state now than when on liver extract therapy previously, without acid.

All three of the patients who developed evidence of neurologic relapse probably ate inadequately. Whether this contributed to the relapse cannot be stated, but it is not unlikely that inadequate diets may contain an insufficient amount of some substance, other than folic acid, which is able to prevent the onset of neurologic relapse.

D. Patients Sensitive to Liver Extracts.—Folic acid was given to two patients who, because of sensitivity to liver extract, had been maintained on oral medication with liver-stomach (Extralin, Lilly). One of these patients (Case 31) was referred to previously. The other (Case 32), a 77-year-old white woman with addisonian pernicious anemia, also had marked sensitivity to injections of liver extract and was subject to severe bronchial asthma. She had been taking Extralin (Lilly), 9 capsules daily, for several years. On this therapy the erythrocyte and hemoglobin levels (Table III) were not optimal. After institution of folic acid therapy there was considerable rise in the blood values. The patient experienced no subjective improvement, however.

Attempts at desensitization of these two patients were made without success. It has been our experience in general that liver extract sensitivity is apparently an organ sensitivity rather than a species sensitivity, as has been suggested.[13] It is also not uncommon, in our experience, to find that oral therapy with liver or stomach preparations may fail to maintain adequate blood levels. As demonstrated by the two patients cited, folic acid may well prove to be a valuable form of therapy in such liver extract-sensitive patients. However, in the presence of neurologic manifestations, additional oral liver therapy would be indicated.

III. DISCUSSION

Discussion has begun to appear concerning the relative ability of folic acid and liver extract to produce and maintain hematologic remission. One author, previously cited,[6] believed that his data indicated that folic acid, unlike liver extract, rarely produced a maximal response in reticulocyte and erythrocyte levels, although he believed a combination of folic acid and liver extract was probably superior to liver extract alone. Another publication,[14] in which the effects of liver extract and folic acid were compared, suggested that the response to folic acid may have been somewhat slower than with liver extract but eventually as good. These authors believed, however, that some patients maintained on folic acid required increasing doses as time elapsed.

In the series of cases studied here, there was no indication that folic acid was unable to produce a maximal reticulocyte response when given in doses of

10 mg. or more daily to patients in relapse, with the one exception cited previously. One patient in particular (Case 41), who later showed neurologic relapse, had a reticulocyte response of 40 per cent when the expected theoretic maximal as calculated for liver extract was 22.3 per cent. This response was followed by an excellent increase in red count and hemoglobin to high normal levels and by disappearance of macrocytosis in a period of sixty-one days.

Similarly, during the period of observation of this group of cases, folic acid showed no inability to maintain the erythrocyte count at high levels when given in sufficient amount. "Sufficient amount" is dictated more by interval than by size of dose. Intermittent medication at intervals of longer than two weeks did not consistently prevent hematologic relapse in these patients. Daily oral administration of 10 mg. was effective in preventing hematologic relapse during the period of observation, which indicates that this method of treatment is the one of choice

No toxic or allergic manifestations to folic acid have been observed to date except after rapid intravenous administration of large amounts (150 mg. in 7.5 ml. of solution). Several patients so treated responded with vertigo, syncope, and marked drop in blood pressure. Recovery was always complete within two or three minutes, except for a headache, which persisted up to twenty-four hours. Since intravenous medication has no advantage over oral or intramuscular use and since it has the possible disadvantage of even more rapid urinary excretion, there is no indication for intravenous administration. Whether these reactions were due to high concentration of folic acid in the blood or to impurities present in the solution was not determined.

IV. CONCLUSIONS

1. Forty-one patients with pernicious or allied macrocytic anemia were treated with folic acid for periods up to one year.

2. Of these, twenty-six showed no hematologic relapse, nine had a decrease in erythrocyte count, and six showed an improvement when the results were compared with those obtained during previous therapy with liver extract.

3. All nine who developed hematologic relapse were given intermittent intramuscular injection of either 50 or 150 mg. of folic acid at intervals of three or more weeks.

4. Of the six showing hematologic improvement, as compared with previous liver extract therapy, three were given daily oral therapy, two were given intramuscular injections at intervals of two weeks, and only one was given intramuscular therapy at an interval longer than two weeks.

5. Intermittent intramuscular therapy with folic acid at intervals greater than two weeks cannot be relied upon to maintain hematologic remission with individual doses as great as 150 mg. Daily oral medication of 10 mg. did not allow hematologic relapse in any patient observed.

6. Six patients given folic acid while in hematologic relapse had a satisfactory reticulocyte response and increase in erythrocytes and hemoglobin.

7. One patient was encountered who showed very minimal hematologic response when given daily intramuscular injections of 12 mg. of folic acid.

A prompt and theoretically maximal response ensued when liver extract therapy was instituted.

8. Three of the forty-one patients developed neurologic relapse when the erythrocytes and hemoglobin were at normal levels on folic acid therapy. These patients responded to liver extract therapy.

9. In spite of its inability to prevent neurologic relapse, folic acid has a place in the therapy of macrocytic anemia, particularly in those patients who are sensitive to extracts of liver. In such instances, its combination with oral liver extract preparations might be desirable.

10. Toxic or allergic manifestations to folic acid have not been observed except for mild nitritoid reactions following rapid intravenous administration.

11. Because of neurologic relapses occurring in patients treated with folic acid, this drug cannot be considered a complete substitute for liver extract, and liver extract, or liver extract combined with folic acid, must remain the treatment of choice for pernicious anemia.

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PERITONEAL IRRIGATION IN THE TREATMENT OF RENAL FAILURE DUE TO TRANSFUSION REACTION

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THE recent report of successful peritoneal irrigation in a patient suffering from renal failure following the administration of sulfathiazole[1] prompted us to use a similar method in the treatment of a man who developed severe renal damage following a transfusion reaction. During the course of peritoneal irrigation, the patient developed generalized edema. Death occurred on the eighth day following transfusion. Because the Tyrode's solution used for irrigation contained a higher concentration of sodium chloride than did plasma, it seems possible that the generalized edema was due to salt retention from the irrigating fluid. Because of the appearance of edema during the course of therapy, this patient is of particular interest.

CASE REPORT

The patient was a white man, 29 years of age, with tuberculosis of the eighth and ninth thoracic vertebrae and a large paravertebral abscess. He was afebrile and the general condition was good. An intravenous pyelogram and repeated urinalyses were normal.

On May 21, 1946, spinal fusion was performed, and during operation a blood transfusion of 500 ml. was given. Cyanosis, unrelieved by oxygen administration, was observed by the anesthetist during and after the transfusion. The total urine output for the following days is shown in Table I.

The oliguria was first noted twenty hours after transfusion and 2,000 ml. of 5 per cent glucose in distilled water and 1,000 ml. of ⅙ M sodium R lactate were given intravenously. A specimen of venous blood showed no gross evidence of hemolysis and the icterus index was 20. On the supposition that the renal suppression might be reflex in origin, efforts were made to stimulate renal excretion by the intravenous administration of 100 ml. of 25 per cent glucose and 40 ml. of aminophyllin (4.0 Gm.). A high spinal anesthetic and continuous gastric suction were also used. Two days after transfusion the incompatibility of the transfused blood was established. The donor was found to be Group A and the recipient Group O. The patient's blood nonprotein nitrogen gradually became elevated (Fig. 1, Section B) and attained a level of 126 mg. per 100 ml. on May 25 (four days after transfusion). The patient was drowsy, restless, and irritable and the abdomen was distended. Peritoneal irrigation with Tyrode's solution was instituted at 3:00 P.M. on May 25. The following formula was used:

	Mg. %
NaCl	800
KCl	20
$CaCl_2$	20
$MgCl_2$	10
NaH_2PO_4	5
Glucose	100
$NaHCO_3$	100

All the ingredients except the sodium bicarbonate were dissolved and autoclaved in 2-liter bottles. The sodium bicarbonate was dissolved and autoclaved separately. The fluid

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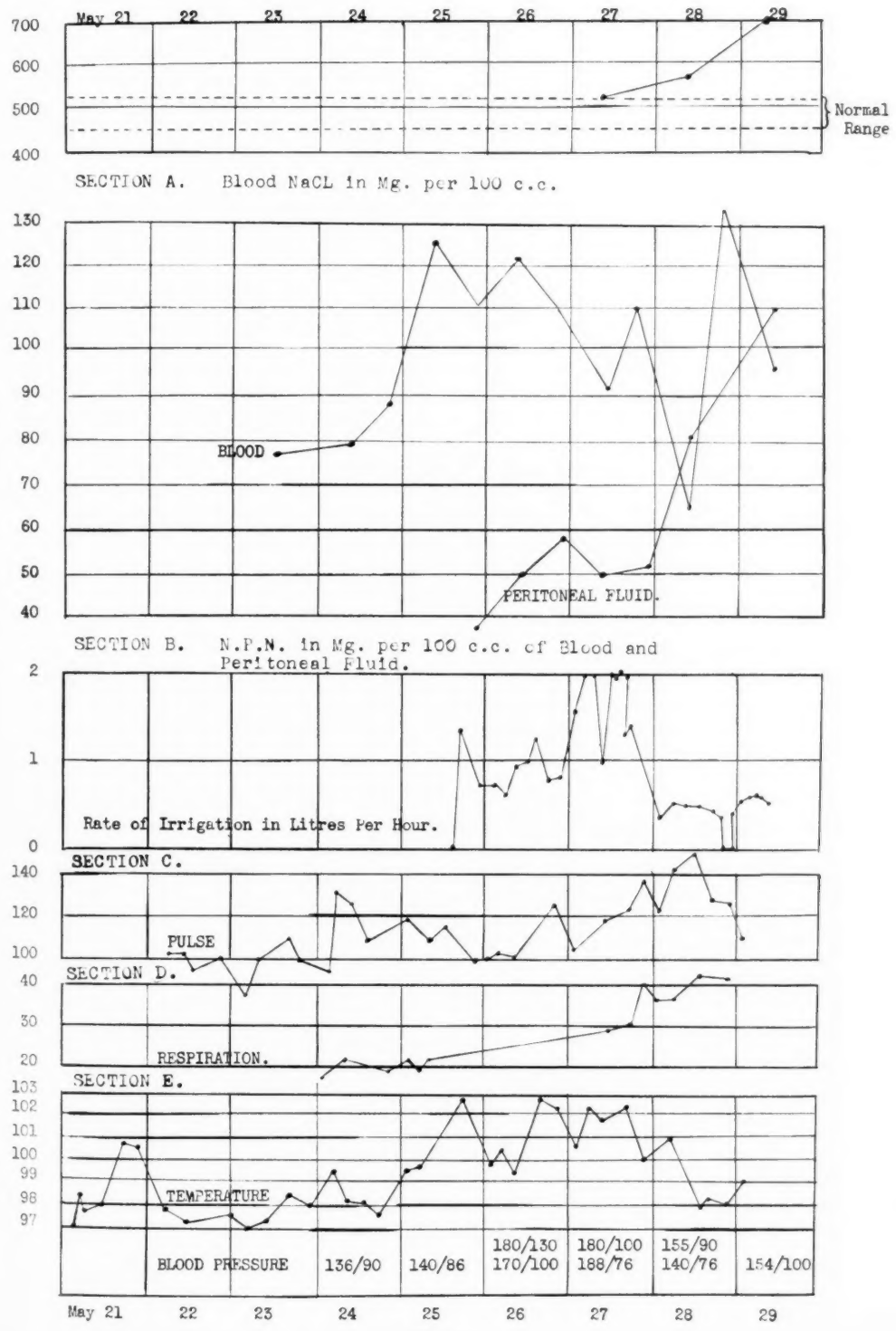
May 21
22
23
24
25
26
27
28
29
700
600
500
400
Normal Range
SECTION A. Blood NaCL in Mg. per 100 c.c.
130
120
110
100
90
80
70
60
50
40
BLOOD
PERITONEAL FLUID.
SECTION B. N.P.N. in Mg. per 100 c.c. of Blood and Peritoneal Fluid.
2
1
0
Rate of Irrigation in Litres Per Hour.
SECTION C.
140
120
100
PULSE
SECTION D.
40
30
20
RESPIRATION.
SECTION E.
103
102
101
100
99
98
97
TEMPERATURE
BLOOD PRESSURE
136/90
140/86
180/130
170/100
180/100
188/76
155/90
140/76
154/100
May 21
22
23
24
25
26
27
28
29

Fig. 1.

TABLE I

DATE	TOTAL URINE VOLUME (ML.)	URINALYSIS
5/22/46	30	
5/23/46	60	Alk.; alb., 3+; R.B.C., 2+; W.B.C., 3+
5/24/46	60	Alk.; alb., 2+; R.B.C., 1+; W.B.C., 1+
5/25/46	60	Alb.; alb., 2+; R.B.C., 1+; W.B.C., 1+
5/26/46	55	
5/27/46	Nil	
5/28/46	30	
5/29/46	Nil	

was warmed in a water bath at 38° C. before being used, at which time the sodium bicarbonate was added together with the following: heparin, 5 mg. per liter; penicillin, 7,500 units per liter; and streptomycin,* 10,000 units per liter. The fluid was admitted to the peritoneal cavity through a mushroom catheter to the left of and above the umbilicus and removed by an abdominal sump suction apparatus directed into the pelvis through an incision in the right lower quadrant. Continuous suction was maintained by a Stedman pump and the suction device was adequate to keep up with any given rate of intake. The greatest rate of flow was 2 liters per hour, the average rate being 1 liter per hour (Fig. 1, Section C). The outcoming fluid was slightly yellowish-green in color and definitely turbid. Peritonitis was guarded against by sterile technique, penicillin dressings around the peritoneal tubes, intramuscular penicillin, and penicillin and streptomycin in the irrigating fluid. The outlet fluid was sterile at all times, and at autopsy there was no evidence of wound infection or peritonitis. The irrigation was maintained continuously except for a lapse of a few minutes on May 27 and for a period of three hours on May 28.

On the morning following the commencement of the irrigation the patient showed considerable subjective improvement. Slight subcutaneous edema of the buttocks and ankles was noted at this time, but the chest was apparently clear. From this time on, the picture was one of slowly progressive generalized edema. Moist râles were heard at the bases of both lungs and on May 27 the patient became dyspneic. On this date a venesection with removal of 500 ml. of blood was performed and 150 ml. of twice concentrated plasma were given without apparent effect. The edema progressed and the patient died on May 29, seven days after the administration of the incompatible blood and four days after the institution of peritoneal irrigation.

In addition to the treatment described, the patient received vitamin C and vitamin B complex. Continuous oxygen by B L B mask was given from May 27, and frothy tracheal fluid was repeatedly aspirated. Intravenous fluids were administered as shown in Table II.

During the course of observation the gastric contents were removed through a Levine tube, but this was tolerated only about one-half of the time. While the tube was out the

TABLE II

DATE	FLUID	AMOUNT (ML.)
5/22/46	5% glucose in distilled water	2,000
	⅙ M sodium R lactate	1,000
5/23/46	5% glucose in distilled water	1,500
	25% glucose in distilled water	100
5/24/46	10% glucose in distilled water	500
5/25/46	10% glucose in distilled water	500
	10% glucose in normal saline	500
5/26/46	Plasma	500
	10% glucose in distilled water	150
	Twice concentrated plasma	150
5/28/46	Nil	
5/29/46	Nil	

*The streptomycin was supplied through the courtesy of Dr. L. E. Ranta, Western Division of Connaught Laboratories, Vancouver, B. C.

patient drank small quantities of fluid and vomited intermittently. The blood carbon dioxide combining power of the patient varied between 40 and 66 volumes per cent during the period of observation.

*Autopsy Findings.**—Autopsy revealed the following: (1) acute nephrosis (transfusion kidneys), (2) pulmonary edema (marked), (3) generalized edema, (4) cardiac dilatation, (5) miliary tuberculosis of lungs, liver, and spleen, (6) vertebral tuberculosis and paravertebral abscess, (7) operation (spinal fusion).

The cause of death was pulmonary edema. The abdominal cavity contained about 100 ml. of thin yellowish fluid. There were no fibrinous adhesions over the intestines and the peritoneal surfaces were smooth and glistening. The two abdominal stab wounds were clean and in no place was there evidence of pressure on the viscera by the drainage tubes. The liver weighed 2,200 grams and was large and dark red in color. Its cut surface showed normal architecture. The spleen weighed 450 grams and had old fibrous adhesions at its upper pole. The right kidney weighed 280 grams and the left, 260 grams. They were large but not tense. The cut surfaces revealed pale yellowish-pink cortices measuring 0.6 to 0.7 cm. in thickness. The pyramids were a deeper pink. No petechial hemorrhages were present. The renal pelves were of normal size and their lining membranes were pale.

The most striking microscopic change in the kidneys was dilatation of all convoluted and straight tubules, with flattening of their epithelium, and the presence of pink-staining fibrillar material in their lumina. Some tubules contained solid, brownish-red amorphous material in this situation. The interstitial tissue in scattered regions was a little loose and edematous and in a few scattered places there was some infiltration with lymphocytes. There was no evidence of renal tuberculosis.

COMMENT

That the irrigation was effective in reducing the blood nonprotein nitrogen is suggested by the large amount of nonprotein nitrogen removed in the drainage fluid (Fig. 1, Section B) which, on May 29, reached a level, in a twelve-hour aliquot specimen, of 110 mg. per 100 ml., and by the decline in blood nonprotein nitrogen following the institution of the irrigation. The blood nonprotein nitrogen reached its lowest level following a period of irrigation at a rate of 2 liters per hour, only to rise again during the time when the irrigation was slowed or temporarily stopped (compare Section B and C in Fig. 1). It seems reasonable to assume that during this four-day period the nonprotein nitrogen would otherwise have risen steadily.

The outstanding feature of the case was the progressive edema and there seems to be no doubt that this was the main factor in causing death. In considering the cause of the edema the following points are important.

Fluid Intake.—The intravenous fluid intake was not excessive (Table II). During eight days of observation the patient received only 6,900 ml., an average of only 860 ml. daily. The oral intake could not be measured accurately enough for record purposes. The patient frequently drank small quantities of water, but also vomited frequently and had a stomach tube (with suction) in place a considerable part of the time. It seems likely that any excess of oral intake over oral output was very small indeed and it may be that the balance was negative.

Fluid Loss.—The respiratory rate was consistently above normal (Fig. 1, Section E) and the patient perspired profusely, causing considerable fluid loss.

*Autopsy performed by Dr. H. K. Fidler, Shaughnessy Hospital, Vancouver, B. C.

The urine output was only 295 ml. in eight days. During this period one enema was given, with good return, and there were three spontaneous and involuntary bowel movements. There was no diarrhea and the stool appeared normal.

Protein Balance.—Protein loss was considerable (Table III) but the effect on the plasma protein was not great.

TABLE III

DATE	PROTEIN CONTENT OF OUTFLOW FLUID (GM. PER 100 ML.)	PLASMA PROTEIN (GM. PER 100 ML.)
5/25/46	0.38	6.2
5/26/46	0.10	5.12
5/27/46	0.06	5.4

Chloride Balance.—On one occasion the patient was given 4.5 Gm. sodium chloride in the form of normal saline. The oral intake was negligible. In spite of this the blood chlorides (expressed as NaCl) showed a progressive rise from 520 to 690 mg. per 100 ml. (Fig. 1, Section A).

In reviewing the foregoing data, it would appear that the edema was not the result of too great a fluid or chloride intake by the intravenous or oral routes. Another possible source of edema-producing fluid was that which flowed through the peritoneal cavity. Its electrolyte content was considerably higher than that of normal blood plasma, and it seems likely that while the nonprotein nitrogenous substances were passing in one direction, electrolytes accompanied by water were passing in the opposite direction and producing hydremia and hyperchloremia.

We can find no other explanation for the rising blood chlorides. In the case reported by Frank, Seligman, and Fine[1] the blood chloride remained high (660 mg. per 100 ml.) and it is noteworthy that pulmonary edema developed in their patient on the third day of irrigation. It may be that if the kidneys had not started to function on the following day, the edema might have progressed. This raises the question as to whether or not Tyrode's solution is ideal.

The normal range of serum chloride (expressed as sodium chloride) is between 570 and 620 mg. per 100 milliliters. The Tyrode's solution used in irrigation contained 800 mg. of sodium chloride per 100 milliliter. Since sodium chloride forms a crystalloidal solution, it would pass readily through the peritoneal membrane until the concentration in the serum approximated the concentration in the peritoneal irrigating fluid.

The assumption that the increased chloride was responsible for the tissue edema is supported by the following passage from Stitt and co-workers:[2] "One of the major functions of sodium chloride is to maintain the normal osmotic pressure in the body fluids. Some unknown mechanism, in which the kidney must play the major role, keeps the electrolyte content of the plasma nearly constant. . . . If chloride is ingested faster than it can be excreted, it does not accumulate in the plasma but passes (as sodium chloride and water) into the intercellular tissue spaces and produces edema."

In view of the foregoing, it would appear logical to modify the formula of the peritoneal irrigating fluid for future cases so that it contains 570 mg. of sodium chloride rather than 800 mg. per 100 milliliters.

CONCLUSION

In a case of renal failure caused by a blood transfusion reaction peritoneal irrigation with Tyrode's solution produced an apparent beneficial effect on the uremic manifestations, but the patient died because of generalized edema.

The suggestion is made that a modification of Tyrode's solution (reduction chloride content) might prevent such edema.

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PERITONEAL IRRIGATION FOR ACUTE RENAL DAMAGE FOLLOWING INCOMPATIBLE BLOOD TRANSFUSION: A DISCUSSION BASED ON THREE CASES

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PERITONEAL irrigation as a procedure for clearing waste products from the body has been attempted on several occasions in the past.[1-4] Recently this procedure has been rejuvenated as a result of improved techniques and results presented by Fine, Seligman and Frank.[5-7] Other reports[8-10] have resulted from the stimulus initiated by these workers.

We have conducted this procedure of peritoneal lavage on three cases of renal insufficiency resulting from incompatible blood transfusion.* Since experience with the first case, there has also been the opportunity to observe the results of different types of management (mentioned later) on additional identical cases and other related types of acute renal damage. The latter will be presented in a review of all known hemolytic transfusion reactions from this hospital since the inception of the blood bank in 1939.[11] An appraisal of the peritoneal irrigation technique resulting from this experience seems appropriate.

TECHNIQUE OF PERITONEAL IRRIGATION

Introduction of Inlet Catheter and Outlet Drain.—The arrangement for irrigating the peritoneum has been in each case identical in its main features to that presented by Fine, Seligman, and Frank.[5, 7] Under local anesthesia (novocain) and through a low lateral McBurney incision a "sump" drain[7, 12] (Fig. 1) was inserted into the right lower quadrant and placed in the right paracolic gutter adjacent to the cecum. The incision was sutured snugly about the upper collar of the "sump" drain. Through a minimal skin incision a thoracentesis trocar was introduced through the abdominal wall of the left upper quadrant two inches below the costal margin. A No. 18 French catheter was introduced well into the peritoneal cavity and the trocar was removed. The skin edges were sutured snugly about the catheter. The entire procedure, conducted in the operating room, was completed within thirty to forty-five minutes.

Irrigating Equipment.—The fluid was allowed to enter through the catheter by gravity and was drained out through the "sump" drain into a 20-liter bottle to which slight negative pressure was applied by a Wangensteen suction or a small electric (aspiration) suction pump. On two cases a bacterial Mandler filter was interposed between the entering fluid reservoir and the intake catheter. No additional pressure was necessary for filtration at the desired rate of flow.

Irrigating Solutions.—Mammalian Tyrode's solution as designated by Seligman and co-workers[6] was most frequently used. On two occasions 5 per cent dextrose in distilled water was deliberately used (see Cases 1 and 2). At other times and for short intervals a sixth-molar sodium lactate and three chlorides (Ringer's) solution was used.

The solutions were prepared in two different manners. In the first case separately sterilized solutions combined prior to utilization were used. In two cases freshly distilled

From William Buchanan Blood Center, Baylor University Hospital, and Southwestern Medical College.
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*Two cases in our hospital, one case elsewhere.

water was collected in 20-liter bottles and the previously weighed solid ingredients were added just before use. The latter was much more practical in view of the interposed Mandler bacterial filter.

To each liter of irrigating fluid were added 25,000 units of penicillin and 1 mg. of heparin. During the irrigation, penicillin also was given intramuscularly (usually 20,000 units every three hours). Two cases received streptomycin by peritoneum and muscle during the latter phases of the irrigation.

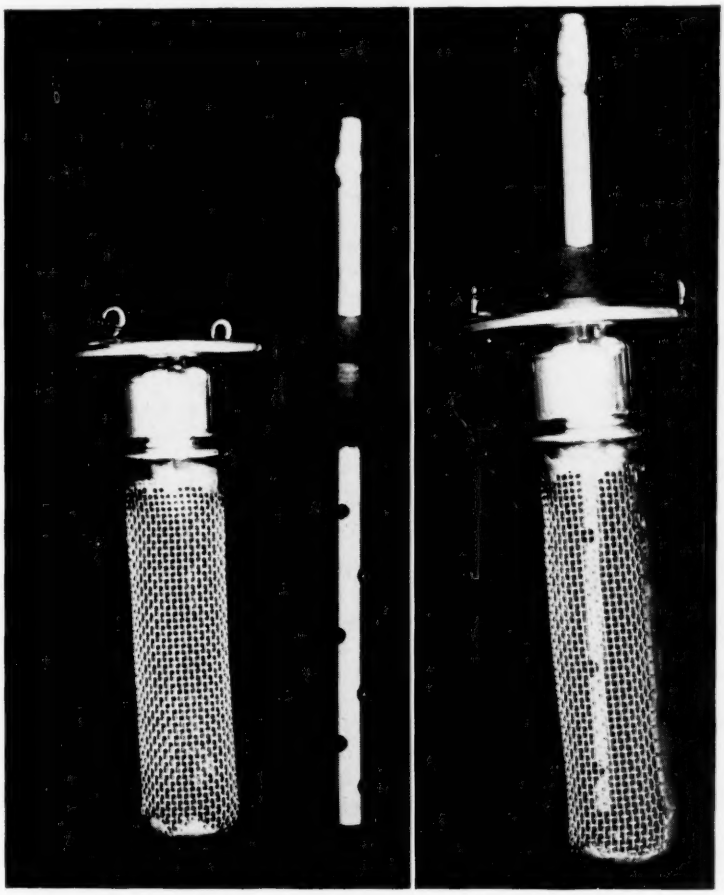

Fig. 1.—The type of "sump" drain used is represented. The abdominal wall was made to fit snugly about the collar and the wire-mesh portion was placed in the right paracolic gutter.

Once started, the irrigation was continuous in two cases and interrupted by a twelve-hour period in one case. The rate of flow was near 1,000 ml. per hour.

Attempts were made to give a high caloric formula by mouth but were not completely successful. Glucose and amino acid were given, but usually not enough for basal dietary demands. Vitamin B fractions, vitamin C, and calcium gluconate were given parenterally. Multiple transfusions were given for anemia.

CASE REPORTS

Whenever possible, a check on the fluid intake by all portals and on the urine output was maintained. A slight but variable leakage always occurred about the "sump" drain, making the measurement of outflow fluid and total urea recovered

somewhat lower than the true value. The urine urea and chloride content were checked daily in two cases. The blood urea,[13] chloride[14] and hemoglobin concentration,[15] and the CO_2 combining power of the plasma[16] were checked frequently. Several serum sodium and potassium concentration determinations[17] were made.

CASE 1.—This case has been discussed elsewhere[10] and only essential points will be mentioned. A 36-year-old white woman was given a transfusion for a moderate blood loss anemia. Through a clerical error type A_1, D-positive* blood was given while the patient was of type O, D positive. A severe reaction developed after 175 ml. were given and the transfusion was discontinued.

There was prominent oliguria for twelve days but during the thirteenth, fourteenth, fifteenth, and sixteenth days a steadily increasing urinary output occurred (see Table 1). During these sixteen days the fluid intake amounted to some 25,750 ml. (17,000 ml. by vein with 70 Gm. NaCl). There was persistent vomiting. The patient's condition deteriorated markedly. There was azotemia (blood urea, 310 mg. per cent), hypochloremia (blood chlorides, 360 mg. per cent), and acidosis (CO_2 combining power, 40.5 volumes per cent). The following signs developed: gallop rhythm, apical systolic murmur, pericardial friction rub, generalized and subcutaneous edema, clonic muscular spasms, blood pressure, 160/110 mm. Hg., and mental confusion.

Peritoneal irrigation over a period of 5.5 days (sixteenth to twenty-second day) was responsible for the clearance of over 76.84 Gm. of urea from the body.

Despite some apparent improvement during the first three days of irrigation (greater mental clearness, diminished muscular jerks, etc.) the patient became more edematous and the cardiac embarrassment seemed more pronounced.† During this time there was a distinct elevation in the blood chloride and serum sodium concentration but the serum potassium concentration remained normal. The hemoglobin concentration of peripheral blood remained at the same level despite prior transfusions of blood. The acidosis became more pronounced. The blood urea concentration was markedly lowered, a lowering not in keeping with the quantity of recovered urea and, therefore, considered as partly a dilution phenomenon. Vomiting and retching continued. It became quite obvious that notwithstanding an already existing extracellular water-salt overload, water and salts continued to be absorbed from the peritoneal surfaces, thus accentuating an already existing complication.

Dramatic beneficial effects in this case were associated with the washing of the peritoneal surfaces with 5 per cent dextrose in distilled water at this time for a continuous twenty-four-hour period. The recovered solution had a composition of 2.5 per cent dextrose and 0.42 per cent NaCl. By this means alone some 60 to 70 Gm. of NaCl were removed from the body. This correction of the salt overload was concomitantly associated with a copious diuresis. A subsequent transient oliguria developed (partly related to water deficit) which, when corrected, was followed by recovery.

Peritonitis due to *Proteus morgani* was treated with streptomycin. It is regrettable to report the development of vestibular damage which did not become evident until after the patient was discharged from the hospital. Otherwise the patient has recovered.

Comment.—Certain distinct complications occurred during the irrigation period: (1) an accentuation of the extracellular overhydration (pulmonary and subcutaneous edema), (2) an accentuation of the extracellular salt overload, (3) the development of severe acidosis, and (4) peritonitis without gaseous distention. Retching and vomiting continued until the catheter and drain were removed.

In the light of subsequent experience gained with these and related cases since the time of the report of this first case, additional interpretations seem

*The antigen D of the Fisher-Race Rh concept.[18, 19]

†A digitalis effect disappeared when the drug was discontinued.

TABLE I (CASE 1)

DAY	BLOOD (MG. %) UREA N	UREA	CO_2 C.P (VOL. %)	BLOOD Cl (MEQ./L.)	SERUM Na (MEQ./L.)	SERUM K (MEQ./L.)	PLASMA PROT. CONC. (GM. %)	HB. (GM. %)	DAYS	FLUID INTAKE (APP.) (ML.)	URINE VOL. (ML.)	URINE UREA (GM.)	URINE Cl (NaCl) (GM.)	PERIT. FLUID VOL. (ML.)	PERIT. UREA (GM.)	EMESIS VOL. (APP.) (ML.)	B.P. (MM. HG)
2	63	135		86.2			5.7	10.8	1-4	5,500	335					1,420	120/ 70
7-8	107	229	58	67.6				9.2	5-8	7,600	575					2,120	134/ 80
12	115	246	48	64.2				10.4	9-12	6,000	1,935					2,620	140/ 80
16	150	310	49	60.8					13-16	6,650	6,600					1,300	160/110
17	88.2	189	46					10.2	17	1,925	2,300	5.2	3.3	11,900	15.9	1,020	150/100
18	100	214		93			6.7		18	2,100	2,130	12.9	8.5	16,000	16.48	2,110	150/100
19	83	178	31	105	150	4.84		10.8	19	2,210	1,725	11.4	6.8	17,800	13.6	900	130/ 85
20	79	169	36	94.3					20	2,125	4,375	15.0	8.2	8,400	5.9	1,310	125/ 80
21	56	120	30	71					21	2,475	2,475	11.3	1.5	19,865	11.6	80	100/ 70
22	50	107	30	74.3				15.8	22	2,840	1,750	10.0	1.3	18,840	13.36	900	120/ 82
25	43	92	56	74.3				10.4	23-28	25,670	20,565	156.7	35+			2,750	120/ 85
29	37	79	54	67.6					29-34	15,600	17,770	133.8	24.3			600	
35	20	43		72.7	142	4.1	6.6	15.0	35-40	19,700	16,585	120.3	35.2				
46	15	32	58	81			7.1		41-46	17,600	21,820	103.8	42.5				

Peritoneal irrigation was conducted in the interval between the seventeenth to twenty-second day. Notice the recovery of substantial urea, but at the same time a marked decrease in the CO_2 combining power of plasma and a prominent increase in the blood chloride concentration. The chloride concentration was lowered after the use of the glucose solution as irrigating fluid, but the acidosis persisted.

The average normal blood chloride concentration by the method used is 84.5 meq. per liter (or 500 mg. per 100 ml. of NaCl). The fluid intake volume is not completely accurate since at times estimations of the oral intake were made. The tendency was for these figures to be conservative rather than excessive. The intravenous fluid intake was always accurately known. The urine chlorides are listed as grams of NaCl. This is purposely done in order to compare the figures with the grams of urea in the urine.

The various abbreviations are self-explanatory.

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justified. The usual time interval for kidney regeneration (structural and functional) had already elapsed by the time the irrigation was commenced. Extraneous factors such as the extracellular salt overload seemed to have interfering influences on renal function as reflected by the occurrence of diuresis and recovery when the salt-removing procedure was instigated. Such reasoning suggests that mostly extrarenal rather than truly renal causes were dealt with by the irrigation procedure in this case.

CASE 2.—This patient, a thirty-year-old white woman, had extensive tuberculosis of the right lung for which a thoracoplasty operation (first and second stage) was performed. She was well nourished.

Unmistakable hemolytic transfusion reaction occurred through no fault of anyone concerned. The patient's blood was typed on many occasions and each time was found to be of type A_1, D positive. All donors giving blood to this patient were rechecked and each was found to be of type A_1, D positive.*

	ANTI-C	ANTI-D	ANTI-E	ANTI-c
Patient	+	+	+	+
Donor 1	+	+	−	+
Donor 2	+	+	−	+
Donor 3	−	+	+	+
Donor 4	+	+	−	−
Donor 5	+	+	+	+
Donor 6	+	+	−	−
Donor 7	+	+	−	+
Donor 8	+	+	+	+

Uneventful blood transfusions were given during the first phase thoracoplasty. During and after the second phase operation, two weeks later, transfusions were followed by hemoglobinuria, jaundice, and the typical type of renal insufficiency. Multiple attempts to demonstrate antibodies in the patient's serum (agglutinating, "blocking," and the "third order" types[20]) were fruitless. The subsequent autopsy demonstrated typical renal lesions of incompatible transfusion type (Figs. 2 and 3).

While under anesthesia (pentothal-cyclopropane-oxygen) during the second phase thoracoplasty the patient received 500 ml. of blood and 500 ml. of 5 per cent dextrose solution. For two hours following the operation the blood pressure remained near 80/60 mm. Hg. Following 500 ml. of dilute plasma the blood pressure became 104/70. At this time (three hours after operation) an additional 500 ml. of blood were given. Nausea and vomiting appeared but the blood pressure remained 114/80. Nine hours after the operation 200 ml. of very dark urine were voided.

Oozing of blood from the operative site began and the blood pressure dropped to 78/50 mm. Hg. After 500 ml. of dilute plasma the blood pressure was 105/70 (nineteen hours after the operation). Under cyclopropane anesthesia the wound was reopened and packed while the patient received 750 ml. of blood, 1,000 ml. of 5 per cent dextrose, and 500 ml. of dilute plasma (blood pressure, from 85/60 to 126/90). At this time 90 ml. of dark brown urine were voided and the patient was distinctly jaundiced (see Table II for icteric index).

The urine specimens during the second twenty-four hours (110 ml.) were likewise brown in color and contained much brownish granular pigment. Because of an alarming lowering of the hemoglobin concentration (5.6 Gm. per cent) the patient was given transfusions on the tenth, eleventh, and twelfth days amounting to 900 milliliters. Again the icteric index was elevated (additional hemolysis).

By summary then, this patient received 2,650 ml. of blood in a twelve-day period, much of which was rapidly hemolyzed through undemonstrated mechanisms.

It was decided to commence peritoneal irrigation as soon as renal insufficiency became definite and severe. We had in mind circumventing advanced renal insufficiency. The total urine volume for a little over three days was 300 to 400 ml., the blood urea concentration was 214 mg. per 100 ml., and, accordingly, the irrigation was initiated.

*The patient's and donors' red cells gave the following results with anti-C, anti-D, anti-E, and anti-c sera of Race.

Peritoneal irrigation was conducted with 5 per cent dextrose in distilled water during the first seven hours as a means of checking the ability of this solution to wash out salts by this technique (refer to Case 1). During this time the solution entered as 5 per cent dextrose and was recovered with the following concentrations: dextrose, 3.9 per cent; chlorides, as NaCl 0.21 per cent; sodium, 91 mg. per 100 ml.; potassium, 8 mg. per 100 milliliters. Thus, in the 5,100 ml. actually recovered at this time the following bulk quantities were measured: 10.7 Gm. of NaCl, 4.64 Gm. of sodium, and 0.4 Gm. of potassium. Additional evidence was gained

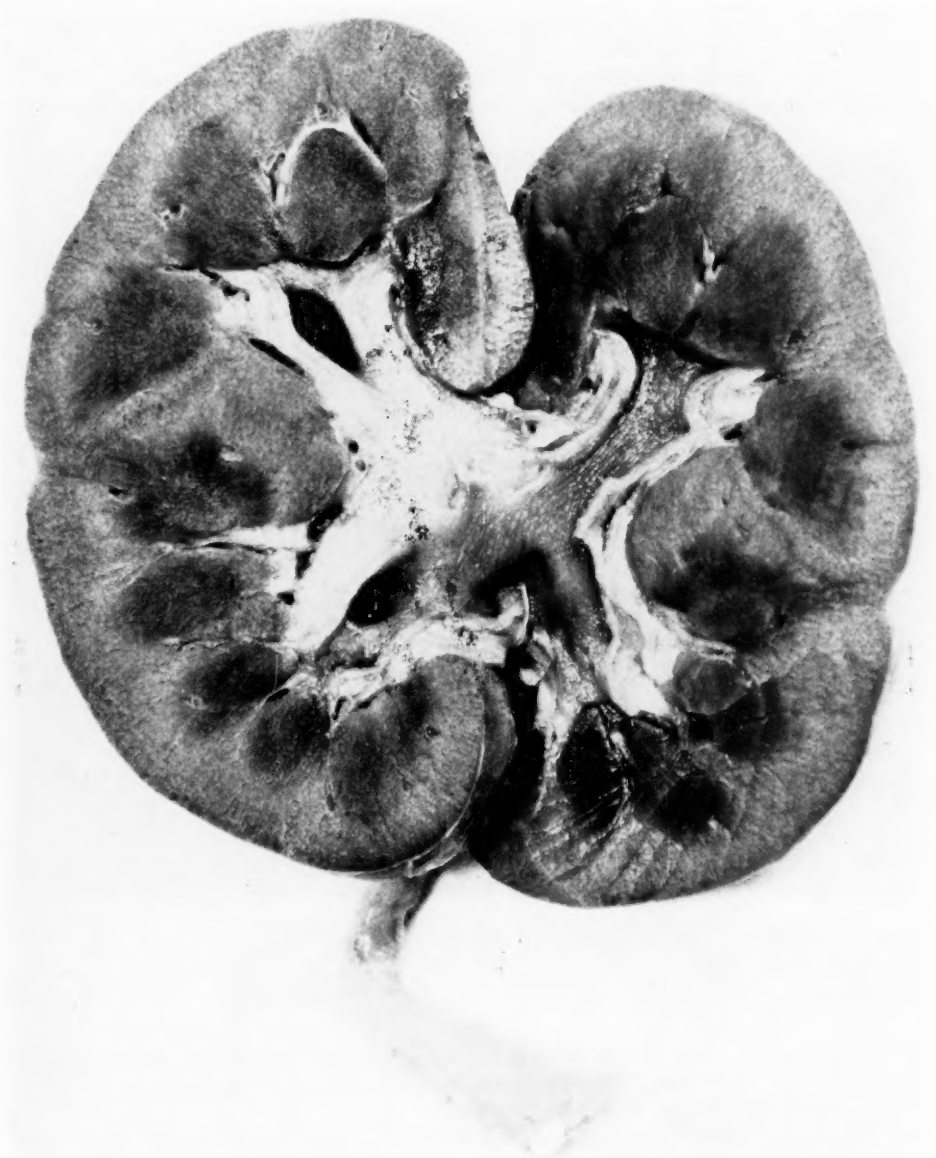

Fig. 2.—The gross picture of a kidney from Case 2. The weight was twice normal and the cut surface bulged markedly. The medulla was dusky and striated due mainly to peritubular hyperemia. The cortex was light in color.

that this procedure can wash out substantial salt just as in the Darrow-Yannet experiment.[21] At the same time some 77 Gm. of dextrose were absorbed.

The irrigation was continued for seven and one-half days. Mammalian Tyrode's solution was used 75 per cent of the time; lactate-Ringer's solution, 14 per cent; and 5 per cent dextrose, 11 per cent. Table II contains the data on this case. The entire amount of urea measured in the outflow fluid was 115 grams. The azotemia abated considerably but again a severe acidosis developed, and some increase in the plasma chloride and sodium concentration occurred.

Most of the 4,025 ml. of fluid given on the first and second days consisted of blood and dilute plasma given to combat blood loss and hypotension. From the third to the eleventh day (irrigation period) the fluid intake, other than by peritoneum, amounted to 13,030 ml. and was given to equalize the insensible water loss and slight urinary output. On this regime

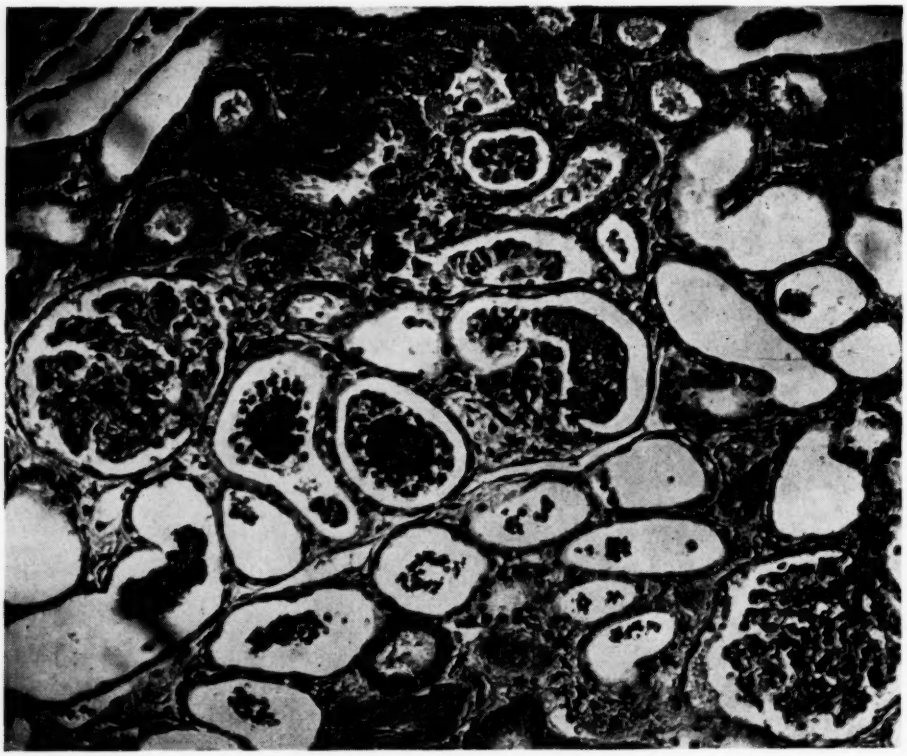

Fig. 3.—Section of kidney shown in Fig. 2. The glomeruli are of normal size and cellularity, but there is protein precipitate within the capsular spaces. The proximal segments are shown in the upper portion and display hyaline droplets in the cytoplasm. The distal segments are lined by thin regenerated epithelium and contain granular heme casts. In the center damaged epithelium has sloughed into the lumen and surrounds a heme cast (brownish granular) in the manner of a collar.

the patient became edematous in the subcutaneous zone, but there was no demonstrable pulmonary edema. Such circumstances can be explained best by considering that water and salts were absorbed through peritoneal surfaces.

The hypertension which began on the third day persisted throughout the irrigation period. During the first three days sodium bicarbonate was given (1 Gm. every four hours) but this was discontinued when the edema was noticed. Within two days a severe acidosis associated with Kussmaul breathing appeared. In required 32 Gm. of sodium bicarbonate in twenty-four hours to alleviate the acidotic breathing. The acidosis was modified but remained severe.

TABLE II (CASE 2)

DAY	BLOOD (MG. %) UREA N	UREA	CO_2 C.P. (VOL. %)	PLASMA Cl (MEQ./L.)	SERUM Na (MEQ./L.)	SERUM K (MEQ./L.)	ICTERUS INDEX	HB. CONC. (GM. %)	FLUID INTAKE (ML.)	URINE VOL. (ML.)	URINE UREA (GM.)	URINE Cl (NaCl) (GM.)	PERIT. FL. VOL. (ML.)	PERIT. UREA (GM.)	B.P. (MM. HG)
1-2	46	99							4,025	290	1.9	1.1			
3	65	139	63	84.5			82.8		400	110	0.7	0.45			120/80
4	100	214	62	86.2	153	3.0	173	9.5	200	115	0.54	0.34	5,100	6.8	120/80
5	107	229	56	71	132	6.2	160	9.5	1,300	225	0.48	0.4	22,200	26.6	
6	101	216	34	82.8			157		2,100	140	0.42	0.5	29,200	25.8	132/84
7	89	191	42	91.3			150		1,450	255	0.6	0.26	27,150	21.8	134/84
8	81	173	22	98	142	4.2	100		2,440	235	0.49	1.1	26,200	14.0	145/75
9	77	165	36	101			92.8	5.6	2,000	300	1.25		20,000	9.0	160/90
10							69	7.2	1,480	250	1.0		20,300	9.0	180/96
11	57	122	26	101			95.7	8.2	1,660	245	0.26	1.2	3,150	2.0	170/90
12	68	145	43	88			97.5	8.6	100	230	0.64	0.86			
13	79	169	38	84.5			111.6		520	620	2.0	2.1			
14	86	184	50	84.5	134	4.9	122.5		1,200	460	1.37	1.7			
15	94	201	43	88					80	475					140/90

The same scheme is used as in Table I. The chloride determination was performed on samples of plasma on each occasion. The normal plasma chloride concentration is usually given as 103 meq. per liter. Notice that a prominent hypochloremia existed at the outset of the irrigation.

Acidosis and increase in the chloride concentration are evident. The fluid intake was accurately measured in this case. After cessation of the irrigation the CO_2 combining power was elevated and the chloride concentration dropped once more.

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Up to the fourth day of irrigation there was little evidence of improvement other than reflected by a lowering of the blood urea concentration, a minimal increase in urinary volume, and a recession of the jaundice. At this time pronounced hemodilution existed (hemoglobin 5.6 Gm. per cent) and three small (300 ml.) transfusions were given. That this course was unsuccessful was attested by the recrudescence of the jaundice, the subsequent rather fixed oliguria, progressive weakness, and mental confusion. The course continued downhill with marked gaseous distension, and expiration occurred following a convulsion on the fifteenth day.

On the fifth day of irrigation the outflow fluid became cloudy and showed many strands of fibrin and clumps of leucocytes. A culture at this time yielded a growth of a gram-negative bacillus identified as Proteus. Streptomycin therapy was initiated and continued until the exitus (0.25 Gm. intramuscularly every four hours).

Pertinent features of the autopsy findings are given. There was widespread fibrocaseous tuberculosis of the lungs with nearly complete destruction of the right lung and extensive involvement of the left upper lobe; the left lower lobe constituted the only morphologically intact lobe. Of much interest was the complete absence of evident pulmonary edema. A minimal amount of greenish-yellow fibrinopurulent exudate was found about the catheter and drain wounds but in all other areas the peritoneal surfaces were of normal smooth and glistening appearance. The liver displayed moderate fatty metamorphosis and bile staining. The kidneys were outstanding. They were twice normal in weight (right, 300 grams; left, 300 grams); the cut surfaces bulged prominently; the capsule stripped easily, leaving a smooth surface; the cortex was gray-yellow in color; the medulla was dusky and well striated (Fig. 1). Microscopically the following were noted: protein precipitate in the glomerular capsular spaces, normal-appearing glomeruli, prominent hyaline droplet accumulation in the cytoplasm of the cells lining the proximal segments, focal necrosis of the distal segments with focal inflammatory foci (lymphocytes, eosinophils, macrophages, fibroblasts), many heme casts in the distal segments (brownish granular and eosinophilic granular types), prominent sloughing of damaged cells of the distal segment, regeneration of the distal segment, and interstitial edema. The regenerated distal segments were lined by very flat cells as described by Lucke[22] (Fig. 2). Prominent medullary capillary hyperemia was present.

Comment.—This case presented several points of much interest. In the first place the exact antigen-antibody mechanism causing the hemolysis was not demonstrated, but undoubtedly multiple hemolytic transfusion responses occurred and resulted in a severe type of renal insufficiency. The failure to demonstrate any type of antibody (agglutinating, blocking, developing) in the patient's serum, despite daily checks, was a new experience for this center. It could be that the transfusions cleared the serum of antibodies and that the widespread tuberculosis hindered further antibody production.

A series of interesting points were related to the irrigating procedure. It was reasoned that by initiating irrigation early it would be possible to keep ahead of the deleterious aspects of renal insufficiency and to precipitate an early recovery of the kidneys. It is now clearly apparent that this was a distinct mistake. The usual progress of the renal phase was not altered, as can be appreciated by comparing this case with fourteen others discussed elsewhere.[11] Considerable urea was cleared from the body, but a severe acidosis developed and generalized edema was a continuous threat. It becomes apparent that the difficulties in this case resembled those of Case 1.

By the foregoing remarks it is not meant to imply that the irrigation procedure necessarily caused the fatality. Two other complications were more likely to have been implicated in this sense: (1) overwhelming pulmonary tuber-

culosis demonstrated at autopsy and (2) repeated transfusions with hemolytic response. But it does appear that the procedure was begun much too early, that the usual course of these cases was not altered, and that certain deleterious complications were once more demonstrated.

Finally, this case offered three other worth-while informative features. (1) The washing out of salts and base via the peritoneum by a nonsalt-containing fluid was again revealed. This is merely a clinical application of the Darrow-Yannet experiment[21] and is fraught with possible deleterious fluid shifts under certain conditions (see Discussion). (2) The absence of a diffuse peritonitis following seven and one-half days of irrigation when penicillin and streptomycin were used is in itself worthy information. (3) The morphologic renal picture corresponded with the lesions designated by Lucke[22] as "lower nephron nephrosis" and included sloughing of damaged tubular epithelium and well advanced regeneration of the same by the fifteenth day.

CASE 3.—This case is that of a colored woman, aged 49 years, who developed a prominent hypotension associated with blood loss during a hysterectomy for multiple leiomyomas.* Due to the urgency of the hypotension, unmatched blood was given (500 ml.).

The blood pressure remained low (70/40 to 86/70) for nearly twenty-four hours. The patient had received 3,500 ml. of blood. A laparotomy revealed a hematoma in the pelvis and the oozing of blood; this was controlled and the blood pressure returned to normal. (Hemorrhagic tendencies are not an infrequent complication of hemolytic reactions.)

On the second day the icteric index was 26. The first urine specimen was dark red. Oliguria became marked (for ten days about 100 ml. daily). There was mounting azotemia, acidosis, and hypochloremia (Table III). Vomiting was troublesome. The patient became comatose and developed muscular twitching. The total amount of fluid intake is not known. During the first five days roughly 3,000 ml. were given in addition to the blood given for the hypotension. Between the fifth and tenth day 13,500 ml. of intravenous fluids were given, of which 5,000 ml. were of isotonic sodium salt composition.

TABLE III (CASE 3)

DAY	BLOOD (MG. %) UREA N	BLOOD (MG. %) UREA	CO_2 C.P. (VOL. %)	BLOOD Cl (MEQ./L.)	SERUM Na (MEQ./L.)	PLASMA PROT. CONC. (GM. %)	HB. CONC. (GM. %)	URINE VOL. (ML.)	URINE UREA (GM.)	ICTERIC INDEX	PERIT. FLUID VOL. (ML.)	PERIT. UREA (GM.)
1												
2			55	99			9.8	100?		26		
3								100?				
4								100?				
5	106	227						150?		8		
6	105	225	42					100				
7	168	360	28	86.4		7.1				6		
8	222	475	45.7	72.9	140		11.5	200				
9					140		7.8	100	0.08			
10	198	424	69.8	66.6								
11	220	471	50.4	52.5			6.8	150			17,000	30
12	182	389	50.7	99.9	150	7.6		380	0.9		22,210	22
13	126	270	33.7	116				610	1.36		19,000	19.5
14	106	227		104.7								

The chloride concentration is once more as blood chloride (average normal, 84.5 meq. per liter). The total fluid intake was not accurately measured or estimated.

Again the drop in CO_2 combining power of plasma and the increase in chloride and sodium concentration during the irrigation are evident.

*This case was observed in another hospital by one of us (A. B. S.). It is not included in the fifteen cases of hemolytic reaction reported separately.[11]

During the eighth, ninth, and tenth days the stomach was periodically washed for clearance purposes. It is of interest that on two occasions the urea concentration of the aspirated fluid was determined to be 3.4 and 3.2 mg. per 100 milliliters.* During this period there was a slight decrease in the blood urea concentration, but the CO_2 combining power of the plasma was elevated and the hypochloremia became of extreme proportions. The serum sodium concentration was within normal limits.

On the tenth day peritoneal irrigation was started because the patient did not appear to be improving. During the following three days 71.5 Gm. of urea were cleared from the body by this means. The urine volume output was increasing slightly. The blood urea concentration dropped markedly but there was pronounced acidosis (Table III). The blood chloride and serum sodium concentration were prominently elevated. On the fourth day of the irrigation the patient succumbed to an accident not related to the peritoneal irrigation or to the renal insufficiency.† The ultimate outcome of this case, therefore, is not subject to appraisal in so far as the renal lesion and irrigation are concerned.

Comment.—Again peritoneal irrigation was responsible for the clearance of a substantial quantity of urea from the body, but concomitantly a severe acidosis developed and there was once more evidence of the uncontrolled absorption from the peritoneal surfaces (elevated Cl and Na concentrations).

The autopsy findings demonstrated typical renal lesions. There were heme casts and sloughing of the damaged cells of the distal tubule and evidence of regeneration by the fourteenth day. Again no diffuse peritonitis was encountered.

TABLE IV. UREA CONCENTRATION (MG. PER 100 ML.) BLOOD AND PERITONEAL FLUID

DAY	CASE 1		CASE 2		CASE 3	
	BLOOD	PERIT.	BLOOD	PERIT.	BLOOD	PERIT.
1	310	133.5	214	133	472	176
2	214	103	229	120	389	99.5
3	178	76	217	88.5	269	102.6
4	169	71.2	191	80.2		
5	120	58.4	174	53.4		
6	107	71	165	64.6		
7-8			123	65		

Notice the usual decline in the urea concentration of the peritoneal escape fluid as the blood urea concentration decreased. These concentrations are minor as compared with the normal urine urea concentration.

DISCUSSION

Continuous peritoneal irrigation has been demonstrated again to remove urea from the body. The urea has been used as an index of the clearance of other waste products. The degree of efficiency of this procedure can be appreciated by examining Table IV. The highest urea concentration in the outflow fluid occurred on the first day when the blood urea level was highest.‡ This highest concentration still fell short of the normal urine urea concentration by ten to fifteen times. Extremely large volumes of fluid therefore were necessary in order to clear daily between 15 and 30 Gm. of urea from the body. As the blood urea concentration was lowered there was some correlation with the decrease in the peritoneal fluid concentration.

*At this concentration in order to clear the 30 Gm. of urea removed on the first day of peritoneal irrigation it would have required 1,000,000 ml. of gastric washing fluid.

†While a sternal transfusion was being attempted (hemoglobin, 6.8 Gm. per cent) 3,000 ml. of blood were introduced into the mediastinum and pleural spaces.

‡This feature satisfies the law of partial pressure as applied to osmotic interchanges.

Two major complications were related to the irrigation procedure in each case: (1) a severe acidosis and (2) the absorption of water and salts from the peritoneal surfaces. There were indications that the acidosis was due to the differential washing out of base while neutral salts were absorbed. Generalized edema developed or became accentuated, and the threat of pulmonary edema remained constant.

Peritonitis was demonstrated in each case but was of a focal nature, being localized about the intake catheter and outflow drain. As a result of exudate accumulation about the "sump" drain the recovery of fluid became less and less efficient after the fifth day. Diffuse peritonitis apparently was prevented by penicillin and streptomycin. Gaseous distention was troublesome in one case.

We are familiar with six other cases of peritoneal irrigation for acute renal damage reported in the literature of recent times[7-9]; four of these cases had renal insufficiency following incompatible blood transfusions. The present group brings the total number to seven. In order to appraise this procedure it becomes necessary to review briefly the sequence of events in such cases.

The clinical course of patients experiencing incompatible blood transfusions may be divided into three phases[11]: (1) *hemolysis, hypotension, etc.*, is characterized by a sudden onset with apprehension, tightness in the chest, backache, dyspnea, cyanosis, mental confusion, hypotension, chill-fever, and hemoglobinemia-hemoglobinuria. (2) *Renal insufficiency* is attended by oliguria, heme casts in the urine, hypertension, azotemia, altered chemical pattern of blood (mainly depressed serum sodium, calcium, blood chloride concentration, and CO_2 combining power of plasma), and mounting antibody titer. (3) *Copious diuresis* is accompanied by marked water-salt loss from the body. Experience with these and related cases have led us to the conclusion that each phase requires its own distinctive type of management.[11] During the first phase, blood transfusion of undoubted compatibility should be given to cope with anemia and lowered blood volume, thus decreasing the period of hypotension. During the second phase, forcing fluids seems deleterious because the kidneys are not in a position to handle water and salts. The best results during this period have been obtained by restricting the fluid intake to a conservative estimated insensible loss plus the scanty urinary output (a total usually of 800 to 1,500 ml. daily for six to eight days). A high calorie-low salt formula has been given with this fluid plus water-soluble vitamins. Sodium bicarbonate (4 to 6 Gm.) has been given daily to cope with an excessive lowering of the CO_2 combining power of plasma. This regime is based on the premise that damaged kidneys require time for healing. When the diuresis appears (eighth to fourteenth day) the water-salt loss through this means is replaced. Such losses may require 20 to 40 Gm. of salts daily plus 5,000 to 10,000 ml. of water. As soon as possible the patient is allowed to adjust his own intake and output.

It has been our experience that patients overloaded with water and salt have expired or the onset of diuresis has been delayed (fourteenth to eighteenth day).

Experience with this three-phase management and with the three cases of peritoneal irrigation has led us to a more critical view of the irrigation procedure. The complications of acidosis and water-salt overload occurring during irriga-

tion seem to be the particular deleterious influences that the outlined three-phase management appears to prevent. With the three-phase management the results have been very encouraging[11]; with the irrigation procedure the results have been rather discouraging.

The discouraging features of peritoneal irrigation are not confined to our three cases. A study of the protocols of the two cases presented by Fine, Frank, and Seligman[7] yield evidence of water-salt overload and of no particular shortening of the usual period necessary for recovery. In the case of Reid, Penfold, and Jones[8] it is difficult to accept the interpretation that the removal of 6.4+ Gm. of urea in two days was an effective means of supporting renal function. Moreover, the onset of the diuresis coincided with the usual time for this phase.

The case of Goodyear and Beard[9] had a stated daily intravenous fluid intake of 3,000 milliliters. Over a sixteen-day period such a regime alone is conducive to a fluid overload when renal insufficiency exists. The amount of urea recovered in the outflow fluid within a six-day period amounted to about 15 grams. One wonders how influential was the factor of dilution in the displayed drop in nonprotein nitrogen concentration. The diuresis phase, which was delayed in onset, occurred after the irrigation.

While treating a case with azotemia, the ability of hydremia to lower the concentration of nitrogenous waste products must be kept constantly in mind. Otherwise a spurious sense of improvement may be enjoyed when actually a deleterious water and/or salt overload is in progress.

The complications encountered in the three presented cases during irrigation have ensued while crystalloid solutions approaching the composition of plasma (minus proteins) were being used. A fluid of different composition may prevent these complications but of this we are not aware at present. Hypertonicity of the inflow fluid has been conducive to pain in a few brief trials.

The results herein presented indicate the need for an earlier and sustained replacement of base. Perhaps the measurement of total base in the outflow fluid would indicate the necessary replacement. Even so, the prevention of the absorption of neutral salts and water remains an additional major problem.

The use of 5 per cent dextrose in distilled water appeared beneficial in Case 1 by removing salt from the body. After nineteen days the kidneys were regenerated and ready for the diuresis phase of functioning. It must be emphasized, however, that the removal of salt in this manner when the kidneys are not ready to discard water may be attended by deleterious intracellular water shift as pointed out by Darrow and Yannet.[21]

CONCLUSIONS

1. Experience with these patients following incompatible blood transfusion in whom peritoneal irrigation was conducted for the treatment of renal insufficiency according to described techniques has been disappointing. Substantial quantities of urea (and presumably other waste products) were cleared from the body. However, two major complications, accentuation of acidosis and the absorption of water and salts from the peritoneum, seemed related to the use of crystalloid solutions for irrigation. Such complications are considered

deleterious to the progress of these patients during the renal insufficiency phase and may retard the onset of diuresis. In contrast, an outlined "three-phase" regime seems to oppose these particular complications.

2. Diffuse peritonitis was absent in two cases examined post mortem. Presumably penicillin and streptomycin therapy prevented this complication. Vestibular damage was subsequently observed in one case following streptomycin.

3. The data obtained in the cases herein reported appears to agree with those of other cases of incompatible blood transfusion and renal insufficiency which have been subjected to this procedure and reported in the literature. There seems to be disagreement in the interpretation given these data.

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SERUM PROTEINS IN ACUTE INFECTIONS

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AS TURBIDOMETRIC methods for the estimation of proteins in biologic fluids have been shown to be unreliable,[1] they have been discarded except for rough approximations. During an investigation of the causes of error, it was observed that in certain acute infections a turbidometric method gave consistently low results for serum albumin when compared with those given by other methods. In the following, these experiments are described and certain conclusions reached concerning the nature of serum proteins in infection.

METHODS

Fractionation of Serum Proteins.—

1. Howe's[2] method. The globulin in 0.5 ml. serum was precipitated with 9.5 ml. sodium sulfate (22.2 Gm. per 100 ml.) at 37° C. and separated by filtration.

2. The Aqueous-methanol method of Pillemer and Hutchinson.[3] The globulin in 1 ml. serum was precipitated by 4 ml. methanol reagent at 0 to 1° C. and separated by centrifuging or by filtration. The clear solution was diluted to four times its volume with saline.

Protein Estimations.—

1. Macro-Kjeldahl nitrogen estimation.

2. Biuret method. The color, developed by Fine's[4] method, was estimated in a photoelectric absorptiometer using a green filter (wave length 525 mμ). The instrument was calibrated from the results of parallel Kjeldahl estimations.

Turbidity Methods.—

1. Total serum protein. One milliliter serum was diluted to 100 ml. with saline (0.9 Gm. per 100 ml.). To 1 ml. of this solution 1 ml. water and 3 ml. sulfosalicylic acid (5 Gm. per 100 ml.) were added.

2. Serum albumin. To 0.5 ml. albumin solution (Howe's filtrate or diluted methanol filtrate), 1.5 ml. water and 3 ml. sulfosalicylic acid solution were added. When the serum albumin was high, the filtrate containing the albumin was first diluted with an equal volume of the solution used for precipitation.

3. For the determination of the specific turbidity (discussed later) of serum globulin the precipitated globulin was washed twice with the precipitant used, dissolved in saline or weak soda, made up to twenty times the original volume of serum, and the protein content and sulfosalicylic acid turbidity estimated. For the latter, 0.5 ml. solution was taken and the precipitation completed as in the case of albumin.

The sulfosalicylic acid was always added slowly, with constant shaking, and the turbid solutions allowed to stand in the incubator for thirty minutes at 37° C. After thorough mixing the optical density of the suspensions was estimated with a photoelectric absorptiometer which had been calibrated with a standard suspension of formazine.[5] This was prepared by mixing 14.5 ml. hexamine solution (10 Gm. per 100 ml.) with 14.5 ml. hydrazine sulfate solution (1 Gm. per 100 ml.) and, after forty-eight hours, diluting to 200 ml. with water. The optical densities of dilutions of this suspension were determined in the absorptiometer, and a curve was constructed from which protein turbidity could be read in terms

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of the standard formazine solution taken as 100. The result was multiplied by a factor depending on the amount of serum present in the aliquot used, so that the final figure represented the turbidity produced in a volume of 5 ml. by 1 ml. of 1 per cent solution of the original serum (total protein, 1; albumin, 0.4, if 0.5 ml. albumin filtrate had been used; globulin, 0.4). For all results recorded here a green filter was used (525 mμ wave length).

As there are not large differences between the quantities of protein precipitated on each occasion, a comparable figure may be obtained for each protein by dividing the turbidity figure by the percentage of that protein in the serum. This figure will be referred to as the "specific turbidity" of the protein.

EXPERIMENTS WITH HOWE'S METHOD OF FRACTIONATION OF SERUM PROTEINS

Sera from normal subjects and from patients with acute infections were fractionated and the total protein, albumin, and globulin determined by the biuret method. The turbidity produced in these fractions by sulfosalicylic acid was measured and the specific turbidity of each protein calculated. In a number of cases serum protein values determined by Kjeldahl were used; these always gave similar results.

TABLE I. SPECIFIC TURBIDITY OF SERUM PROTEINS FRACTIONATED BY HOWE'S METHOD (NORMAL AND ABNORMAL CASES)

CASE	SERUM PROTEINS				SPECIFIC TURBIDITY		
	TOTAL (GM./100 ML.)	ALBUMIN (GM./100 ML.)	GLOBULIN (GM./100 ML.)	ALBUMIN / GLOBULIN	TOTAL PROTEIN	ALBUMIN	
1	6.8	3.8	3.0	1.3	6.7	9.6	Normal
2	7.0	4.0	3.0	1.3	6.6	9.5	Normal
3	7.4	4.4	3.0	1.5	6.2	8.5	Normal
4	7.4	4.7	2.7	1.7	6.7	7.9	Normal
5	6.9	4.6	2.3	2.0	6.9	8.3	Normal
6	7.7	5.4	2.3	2.3	6.7	8.2	Normal
7	7.8	5.0	2.8	1.8	6.3	8.9	Normal
8	7.0	4.6	2.4	1.9	6.7	8.5	Normal
9	7.4	5.1	2.3	2.2	6.6	8.7	Normal
10	7.0	4.4	2.6	1.7	6.4	8.4	Normal
Average of normals					6.6	8.7	
11	6.8	3.3	3.5	0.9	5.7	5.7	Lobar pneumonia: Fever 5 days
12	6.8	4.0	2.8	1.4	6.1	5.8	Lobar pneumonia: Fever 4 days
13	7.8	4.6	3.2	1.4	5.3	5.2	Lobar pneumonia: Fever 6 days
14	5.3	2.9	2.4	1.2	5.7	5.8	Lobar pneumonia: Fever 2 days
	6.3	3.8	2.5	1.5	6.1	7.4	Afebrile 16 days
15	4.2	2.3	1.9	1.2	6.0	3.2	Lobar pneumonia: Fever 7 days
16	7.2	3.7	3.5	1.1	6.0	6.2	Appendix abscess
	7.9	4.1	3.8	1.1	5.3	6.9	8 days after drainage
	8.5	4.2	4.3	1.0	6.6	7.8	20 days after drainage
17	6.8	3.3	3.5	0.9	5.4	4.9	Infected hydatid cyst; lung
18	6.5	3.5	3.0	1.2	6.0	5.0	Infected hydatid cyst; lung
	6.5	3.4	3.1	1.1	5.1	4.6	4 days after drainage
	7.6	4.1	3.5	1.2	5.3	6.2	10 days after drainage
19	5.3	2.5	2.8	0.9	6.3	5.3	Amoebic abscess; liver
	5.8	2.9	2.9	1.0	6.0	6.4	Treatment 10 days
	6.8	3.3	3.5	0.9	6.0	7.2	Treatment 17 days
	6.7	3.9	2.8	1.4	5.9	7.1	Treatment 24 days
	7.0	4.2	2.8	1.5	5.8	7.6	Treatment 50 days
20	7.0	3.5	3.5	1.0	4.9	3.6	Amoebic abscess; liver
21	7.0	3.2	3.8	0.8	5.4	5.7	Osteomyelitis; femur
22	6.8	2.5	4.3	0.6	4.9	6.8	Osteomyelitis; face
Average of abnormals (Initial values only)					5.6	5.3	

Results.—From Table I it can be seen that in acute infections the specific turbidity of total protein and of albumin is reduced, the reduction being small in the case of total protein, large in the case of albumin. During convalescence the value of the latter returns toward normal.

The specific turbidity of globulin in normal persons and in patients with acute infection is given in Table II. It is low and nearly constant and shows no change parallel to that of the albumin. This suggests that the reduction in the specific turbidity of the total protein and the much greater reduction in that of the albumin is to be sought in a change in the albumin-sulfosalicylic acid precipitate. Such a change might be chemical and/or physical; if physical, it might be brought about by the presence of some substance in the albumin filtrate.

In a number of cases the albumin in the filtrate was precipitated by 9 volumes of hot acetone-ethanol (50:50), centrifuged, the supernatant fluid poured off, and the precipitate, after being drained, dissolved in weak caustic soda solution. The turbidity produced by sulfosalicylic acid was determined and the

TABLE II. COMPARISON OF SPECIFIC TURBIDITIES OF HOWE'S ALBUMIN AND GLOBULIN FRACTIONS (NORMAL AND ABNORMAL CASES)

CASE	23	24	25	26	27	28	29	30	31	32	33	34	AV.
Specific turbidity of albumin	8.7	8.3	8.3	8.2	7.8	7.6	7.5	6.8	6.3	6.2	5.7	5.2	--
Specific turbidity of globulin	4.6	3.9	4.0	4.7	4.8	4.5	3.9	4.1	4.4	4.4	4.4	4.2	4.3

TABLE III. COMPARISON OF SPECIFIC TURBIDITIES OF HOWE'S ALBUMIN FRACTION BEFORE AND AFTER PRECIPITATION WITH ACETONE-ETHANOL (NORMAL AND ABNORMAL CASES)

CASE	35	36	37	38	39	40	41	42	43	44	45	46
Specific turbidity of albumin	8.5	8.0	7.9	7.9	6.9	6.4	6.1	5.7	5.3	5.0	4.9	4.8
Turbidity (No precipitation) / Turbidity (Precipitation)	1.3	1.4	1.3	1.3	1.4	1.3	1.2	1.4	1.3	1.3	1.5	1.2

TABLE IV. COMPARISON OF ALBUMIN FRACTION SEPARATED BY HOWE'S METHOD AND BY AQUEOUS-METHANOL METHOD (NORMAL AND ABNORMAL CASES)

CASE	SERUM ALBUMIN (GM./100 ML.) SODIUM SULFATE (1)	SERUM ALBUMIN (GM./100 ML.) AQUEOUS METHANOL (2)	(1) - (2)	$\frac{(1)-(2)}{(1)} \times 100$ (3)	ALBUMIN SPECIFIC TURBIDITY SODIUM SULFATE	ALBUMIN SPECIFIC TURBIDITY AQUEOUS METHANOL
47	4.9	4.2	0.7	14	8.9	11.1
48	5.1	4.4	0.7	14	8.7	11.9
49	5.2	4.7	0.5	9	8.6	10.6
50	4.6	3.8	0.8	17	8.4	12.0
51	3.4	2.4	1.0	29	7.8	11.0
52	3.5	2.4	1.1	31	7.8	12.2
53	3.9	2.8	1.1	27	7.5	12.5
54	3.8	2.8	1.0	26	7.4	12.8
55	2.9	1.9	1.0	34	5.8	11.7
56	2.6	1.6	1.0	38	5.0	11.9
57	3.3	2.0	1.3	40	5.0	11.5
58	4.0	2.1	1.9	47	4.4	12.8
Average						11.8

specific turbidity calculated and compared with that of albumin from the same sera without acetone-ethanol precipitation. The figures in Table III show that, while there is a difference in the specific turbidities of albumin which has and which has not been precipitated by acetone-ethanol, these values bear a constant relationship to one another. This indicates that the cause of the low specific turbidity of albumin in acute infections cannot be separated from the albumin by acetone-ethanol and that it is due to a change in the precipitate itself. Such a change might either be the precipitation of varying amounts of some other substance (for example, carbohydrate or lipoid on the albumin-sulfosalicylic acid precipitate) or the presence in the albumin filtrate in varying proportions of two or more protein substances having different specific turbidities. Lipid contamination is unlikely to be the explanation since acetone-ethanol precipitation would probably remove much of this and the relationship apparent in Table III would not exist.

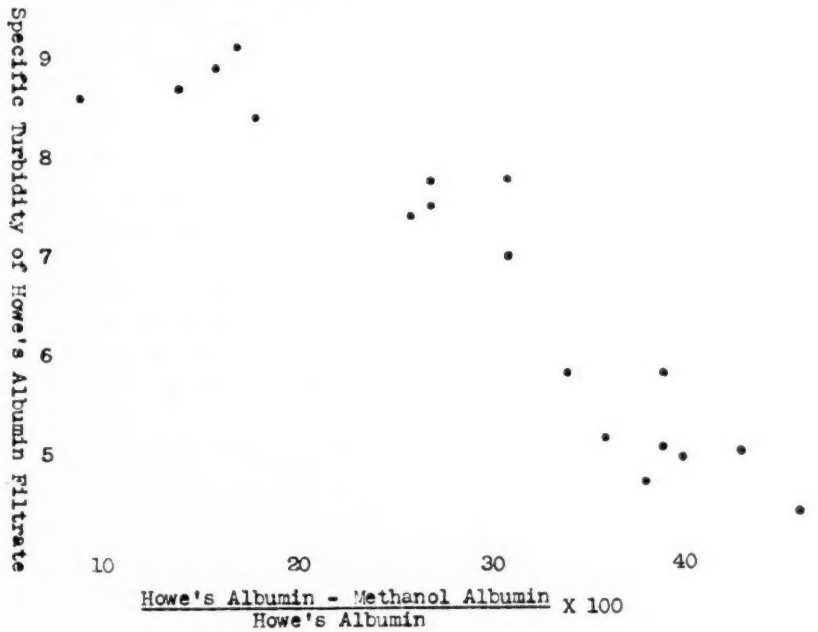

Fig. 1.

EXPERIMENTS WITH AQUEOUS-METHANOL FRACTIONATION OF SERUM PROTEINS

Pillemer and Hutchinson[3] have shown that their method of fractionation gives an albumin:globulin ratio which agrees more closely with that obtained by electrophoresis than does Howe's method. Table IV gives the results of simultaneous serum albumin estimations by the two precipitation methods together with the specific turbidities of both albumin fractions.

It will be seen that sodium sulfate invariably precipitates less protein than does the methanol reagent, a result noted by Pillemer and Hutchinson. The difference is small in normal cases (Cases 47 to 50), larger in cases of acute in-

fection, and represents protein precipitated by the methanol reagent but not by sodium sulfate. If this portion is calculated as a percentage (Table IV, column 3) of Howe's albumin, of which it forms a part, it is seen to vary closely with the specific turbidity of the latter (see Fig. 1).

When the last two columns of Table IV are compared, the specific turbidity of albumin separated by the methanol reagent is seen to be high and, unlike that of Howe's albumin, not to fall in infection. The specific turbidity of methanol globulin was determined in a few cases and found to be low (like Howe's globulin), having a value of 4.7.

Three simple fractions are thus distinguishable: (1) a methanol albumin fraction of high specific turbidity; (2) Howe's globulin fraction of low specific turbidity; and (3) a fraction present in Howe's albumin because not precipitated by sodium sulfate, and also present in methanol globulin because precipitated by the methanol reagent. Since the specific turbidity of methanol globulin is low, this third fraction must have a low specific turbidity too. The reduced specific turbidity of Howe's albumin is therefore explained by the presence in it of this material having a low specific turbidity.

COMMENT

Normally the amount of the third fraction is small, but in certain acute infections it may amount to as much as 40 to 50 per cent of Howe's albumin. Since the change is accomplished within a day or two of the onset of the infection and coincides with a sudden drop in methanol albumin, it is reasonable to suppose that it is formed by the modification of part of the albumin. This "modified albumin," like globulin, is precipitated by the methanol reagent and has a low specific turbidity; but like albumin, it is not precipitated by a 22.2 per cent solution of sodium sulfate.

Tillett and Francis[6] have demonstrated by bacteriologic methods the presence of a nonspecific protein substance, called by them C protein, in the serum of patients with acute infections. This substance was shown by Abernethy and Avery[7] to be associated with the albumin fraction when globulin is precipitated by ammonium sulfate, while by electrophoresis Perlman, Bullowa, and Goodkind[8] have found it in the alpha-1 globulin fraction. These observations suggest that C protein might be the same as the modified albumin described in the foregoing. Modified albumin, however, is not precipitated by 50 to 70 per cent saturation with sodium sulfate as is C protein, according to MacLeod and Avery.[9] Löfström[10] has produced evidence that his nonspecific capsular swelling substance is identical with C protein, but further work is required to confirm the strong presumption that these three substances are one and the same.

SUMMARY

1. In the serum of patients with certain acute infections, a protein is present which differs from normal serum albumin and serum globulin.

2. It appears during the first few days of the infection and is probably formed from serum albumin.

3. This "modified albumin" resembles serum albumin in that it is not precipitated by a 22.2 per cent sodium sulfate solution, but resembles serum globulin in that it is precipitated by a suitable strength of methanol in water.

4. The production of turbidity with sulfosalicylic acid and the estimation of the "specific turbidity" can be used in the investigation of a protein.

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LABORATORY METHODS

A SIMPLIFIED METHOD FOR THE PRESERVATION OF BACTERIA BY DESICCATION IN VACUO

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THE presence of water in liquid form being a sine qua non of active life, the removal of water or, what is almost equivalent from a physiologic standpoint, its immobilization by freezing puts an end to detectable physiologic activity. Life, however, or at least viability, is not necessarily terminated, and this fact is the basis of several methods for the preservation of bacteria in an inactive condition for long periods. Probably the earliest use of this principle was made by Shattuck and Dudgeon in 1912.[1] Processes for preserving bacteria by desiccation after freezing were described by Rogers[2] in 1914 and by Swift[3] in 1921. Brown[4-7] devised a simpler method using only desiccation in vacuo at room temperature, and first showed one of us (M. F., Jr.) how to use it. Brown[7] reported the survival of streptococci, diphtheria bacilli, pneumococci, and other bacteria for periods of from four to twelve years. A modification of his process was described by Leifson[8] in 1936. There is evidence‖ to indicate that the principal function of the vacuum in these processes is that of speeding desiccation, which in turn stops autolysis and other intracellular processes which would prove lethal, although withdrawal of oxygen also doubtless reduces the rate of death of the desiccated cells. Any factors, such as higher and more rapidly produced vacuum, thinner layers of fluid, more effective dehydrating agents, etc., which speed up removal of water, add to the efficacy of the method and probably permit prolonged survival of the more delicate organisms.

In a study of *Serratia marcescens,* Naylor and Smith[9] have shown that high percentages (99 per cent) of cells survive lyophilization if aerated, young cultures are suspended in a solution containing ascorbic acid, thiourea, NH_4Cl, and dextrin at pH 6 to 7. A high vacuum is necessary to high percentage

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‖The older preparations of bacteria listed in this paper were found by one of us (M. F., Jr.) in 1930 to be viable in jars in which the original vacuum no longer persisted after several years of storage at room temperature and transoceanic shipment, unrefrigerated, lasting several weeks in tropical waters. These same jars of cultures, with the same vials, are included in this latest (1946) series of viability tests.

of survival. Attention is called also to the work of Stark and Herrington[10] who published data on differences in resistance of various species to drying in vacuo. Their remarks on free and bound water are of interest. Rahn[11] has given an excellent review of data on the susceptibility and fate of bacteria under desiccation and a discussion of the protective effect of various suspending materials. It seems to be generally agreed that organic colloids have a protective action as a rule, while, in general, contact with oxygen lessens the survival time of the bacteria. In the present study no attempt was made to obtain data on numbers of bacteria surviving after various intervals.

Several processes are in current use in which intracellular water is first immobilized by instantaneous freezing. The effect of this rapid freezing is vitrification rather than crystallization.[12] The water is afterward removed by producing a vacuum in the vessels containing the frozen material. This results in sublimation.[13, 14] The basic principle of this process was used by Sawyer and co-workers[15] for the preservation of yellow fever virus in their early studies, and it proved highly effective. Instantaneous freezing followed by storage at extremely low temperatures (−76° C.) was shown by Turner and associates[16, 17] to be a most effective means of preserving living *Treponema pallidum* and other spirochetes, as well as viruses, in status quo for long periods. More recent studies have shown the applicability of freezing as a means of preserving protozoa and metazoa.[18]

The methods of Swift, of Brown, and of Leifson are not, so far as we are aware, satisfactory for long-time preservation of spirochetes and viruses, as is the method of Turner and co-workers, but a wide variety of other organisms may be preserved by desiccation in vacuo for many years without apparent loss of virulence or alteration of other important properties such as colonial form; motility; hemolysis, pigment or toxin production; morphology; staining and fermentative characters. Although a few reports of remarkable longevity of bacteria on ordinary culture media are to be found in the literature,[19, 20] the usual experience is that stock cultures die off in a few months if not transferred to fresh media. This is inconvenient, time consuming, and expensive and induces alterations in the bacteria, as well as contamination.

This communication is made to describe a simplified method for preservation by desiccation in vacuo, modeled after that of Brown, and to show what may be expected of this simplified method. No claim to greater perfection of final vacuum or permanence of closure is made. The principal advantages are simplicity, availability, effectiveness, and low cost under a variety of conditions. The only special apparatuses needed are a good vacuum pump, such as the "Cenco Hyvac," and a Pyrex vacuum desiccator provided with a one-hole rubber stopper connected to the pump. A one-pint Mason fruit jar, about 100 Gm. of CaCl, and 1 ounce of plasticine are the only materials needed for the desiccation. The bacteria are placed, suspended in sterile rabbit blood, in shell vials 8 by 25 mm.,* previously somewhat less than half filled with

*Arthur H. Thomas Co., Catalogue No. 3648.

"washed and ignited" sea sand, plugged with cotton, and sterilized (Fig. 1, *A*). A good supply of these vials prepared ahead of time makes the preservation of bacteria in vacuo very simple indeed. The necessary steps are as follows:

PREPARATION OF CULTURE

1. The desired organism is cultivated on an agar slant of appropriate composition. After good growth has developed, about 0.5 ml. of sterile, defibrinated or citrated rabbit's blood is placed on the slant and the growth is scraped off the agar and emulsified in the blood. This yields a much heavier suspension than would be obtained by desiccating a broth culture, and the blood, it is believed, when dried on the bacteria, forms a protective coating around them. Heller[21] has recently shown blood to be the best material for desiccation.

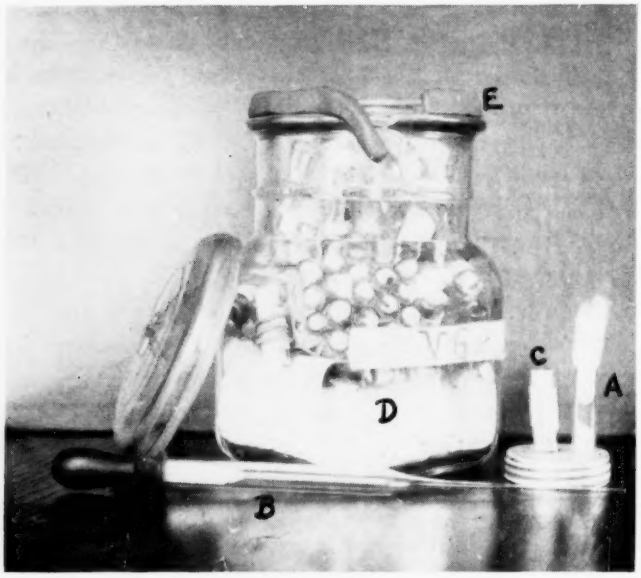

Fig. 1.—*A*, Sterile vial; *B*, pipette; *C*, blooded vial; *D*, cotton; *E*, plasticine.

2. A capillary pipette with 1 ml. rubber bulb (Fig. 1, *B*) is used to take up the blood and transfer it to a sterile shell vial containing sand. The tip of the pipette containing the blood is gently worked downward through the sand until it is at the bottom of the vial and the blood is then made to rise slowly through the sand, thoroughly wetting it by moving the tip of the pipette about gently. Keeping the tip of the pipette at the bottom of the vial, one now withdraws as much of the blood as possible, leaving the sand grains coated with the bacteria-bearing blood. If too much loose blood is left in the vial, it forms a hard, solid mass when dried, which is very difficult to break up when making subcultures from the vial. It is well, therefore, to separate the sand grains somewhat by pushing them partly up the inner side of the vial with the tip of the pipette before finally removing it, so that the grains will not be glued together in a solid mass but can be taken out in small clumps

for subculture. When the sand has thus been "blooded" and the excess blood removed, the cotton plug is replaced.

3. The plug is pushed down in the vial until the upper half of the vial is full of cotton. With scissors, the protruding portion of the plug is clipped off and the tip of the vial is singed so that no cotton fibers protrude (Fig. 1, *C*). Otherwise they may cling to various objects and be pulled out. India ink labelling, covered with nail polish, is convenient. Adhesive tape may also be used as labels. Gummed paper labels are unsatisfactory.

Desiccation.—

1. In the Mason jar, from which the wire clamp has been removed, place about 100 Gm. of technical or C.P. granular calcium chloride. Over this place a ball of cotton sufficient to hold the calcium chloride in place (Fig. 1, *D*). On the cotton place the labelled vials.

Fig. 2.—Type of desiccator and arrangement of moisture traps and manometer.

2. Roll some of the plasticine on the table into a cylinder with the diameter of a lead pencil (about 8 to 10 mm.) and just long enough to form a complete circle around the uppermost ledge of the jar. It is convenient to determine the proper length for the cylinder of plasticine beforehand by making two marks on the table.

3. Put the plasticine in place on the jar (Fig. 1, *E*) and push it into the depression around the top, molding it about 5 to 8 mm. above the top and flaring slightly outward. Place the lid on the plasticine and press it down so that the bottom of the rim of the lid is about 2 mm. below the level of the topmost rim of the jar. Smooth the plasticine all around and make sure that the closure is complete.

4. Insert a needle through the plasticine seal, pointing it slightly upward so that it will pass between the rim of the jar and the lid This leaves an opening about 0.5 mm. in diameter for the escape of air. Be sure the hole is patent.

5. Place the Mason jar inside the vacuum desiccator, close the desiccator tightly, and draw the highest vacuum possible (Fig. 2). It should be at least within 0.1 mm. of barometric pressure. A folded towel should line the desiccator to prevent breakage should the jar topple over.

6. Stop the vacuum pump and, with a *sudden* jerk, withdraw the stopper of the desiccator so that there is a quick, copious inrush of air. The lid of the Mason jar is thus pressed down, occluding the needle hole and firmly squeezing the plasticine ring. The vacuum in the jar will be within a few millimeters of that originally present. This may be verified if desired by including a small, siphon, mercury barometer in the Mason jar before closure. They are not essential but are a useful guide at first. The construction and use of these barometers were described by Brown[4] and Leifson.[8]

Fig. 3.—Method of handling knife in opening the evacuated jar.

Recovery of the Culture.—

1. A tube of broth is prepared by adding to it a drop of sterile defibrinated blood and a drop of sterile 10 per cent aqueous dextrose solution. Other media, appropriate to the bacteria involved, may be used.

2. The effect of the vacuum in the jar is felt when an attempt is made to remove the cover. Force the blade of a large, strong knife (using the portion of the blade close to the handle) between the edge of the lid and the shoulder of the jar. Rotate the knife on its long axis, lowering the back of the knife and keeping the blade firmly pressed between cover and jar (Fig. 3). A screw-

driver blade can also be used but may chip or crack the lid. A sudden hiss signalizes the destruction of the vacuum. Having removed the lid, scrape the plasticine together to smooth the surfaces and to use again.

3. In preparing to recover the culture, partially remove the cotton stopper from the vial with a fine-pointed forceps. After this, handle the cotton stopper as though it were in a diminutive culture tube.

4. A fairly rigid wire is used to loosen the sand grains and to transfer a few of them from the vial to the broth. A two-inch length of No. 14 iron wire or an unfolded paper clip fastened to a needle holder will serve this purpose very well. After flaming the wire, allow a few moments for it to cool; then plunge it first into the tube of broth to be used for the subculture. This cools and wets it.

5. Then introduce the wetted wire into the shell vial. By moving it about, a few grains of sand are loosened. These adhere to the wet wire and are transferred to the blood-dextrose broth. It is best to transfer about a dozen grains.

6. The plug is replaced in the vial and it is resealed in the Mason jar as described. The blood-dextrose broth is incubated appropriately. Occasionally a longer-than-usual period of incubation (for example, forty-eight to seventy-two hours) is necessary.

RESULTS

During the period from 1925 to 1928 and at various subsequent times, one of us (M. F., Jr.) preserved, by the method described, several hundred cultures of various species of bacteria. Many of these were taken from the vacuum desiccating jars and tested for viability and cultural properties in 1930 and 1936, but no report was made on the results of those studies. The present report includes the 1936 data and also results of tests of the same identical cultures, and of some others, made in 1946. Thus information is available on the survival of several species of bacteria when desiccated in vacuum for periods ranging from about one to approximately twenty-one years.

The data may be briefly summarized as follows: of 42 strains of beta type hemolytic streptococci, only 6 failed to grow after eighteen years. All but 2 of 20 strains of *Brucella bronchiseptica* grew after nine years and 1 lived seventeen years. Four strains of alpha type streptococci lived five years; 3 tested after eighteen years were not viable. Of 8 strains of pneumococci, 5 strains lived five years, 1 for nine years, 2 were not viable after nine and seventeen years, respectively. Six strains of *Streptococcus faecalis* and *Streptococcus liquefaciens* lived seven to nineteen years.

Of 69 strains of the enteric group, survival of *Eberthella typhosa* and Salmonella and Shigella species was generally noted after seven years, many strains survived nine years, and 1 strain of *Eberthella typhosa* was alive after eighteen years. Proteus, Aerobacter, and Escherichia and paracolon strains in general lived three to nine years. All of 24 Staphylococcus strains were viable after nine years and all of 15 tested survived nineteen years. Hundreds of strains of *Corynebacterium diphtheriae* of all types have survived thirteen years.

Genera generally surviving at least five years are Serratia, Microbacterium, Pseudomonas, Gaffkya, *Brucella abortus* (1 strain only four years), *Spirillum rubrum*, and Lactobacillus. Species not surviving one year were pathogenic Neisseria (six weeks to three months), *Brucella melitensis* and *Brucella suis* (three months), *Hemophilus pertussis* and *Hemophilus influenzae* (six weeks or less). One strain of Parapertussis bacilli tested after six weeks was found viable.

It may be said of all surviving organisms that the principal distinguishing characters remained unaltered in any respect. Apparently these organisms had been wholly unaffected by their long dormancy.

It should also be emphasized that, with the few exceptions noted, all of the organisms listed here will remain viable without difficulty for at least a year, thus tiding over intervals between class demonstrations very conveniently. They also remain viable in spite of repeated opening and reclosing of the jars. These facts are perhaps of more practical interest than the survival of some species for twenty-one years or more. When stock cultures are constantly in use, it is more convenient, of course, to maintain them on slants in a vegetative state.

SUMMARY

A method of preservation of bacteria in vacuo with simple materials is described. It is evident that, for many species, the method is a desirable substitute for maintenance of vegetative stock cultures and has many important advantages, such as simplicity, economy of time, labor, refrigerator space, and materials, as well as stability and portability of the cultures. A few species, such as pathogenic Neisseria and Hemophilus, did not survive long under the conditions described, but many others survived for periods ranging from one to twenty-one years.

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DESCRIPTION OF APPARATUS AND METHOD FOR OBTAINING NASAL WASHINGS

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AT THE beginning of an investigation of the etiology of minor upper respiratory diseases, it became evident that a new method was required for obtaining nasal washings. After several attempts at collecting washings by the usual method (instilling material into the nostrils, gargling, and expectorating), the unpleasantness experienced by the donor was of sufficient magnitude to warrant investigation of a new method.

It is known that the anatomy of the nose and nasopharynx is such that material instilled into one nostril can be evacuated from the other by suction without interfering with oral respiration. This is the basis for the "nasal douche with suction," "Nichols nasal siphon," and similar therapeutic aids which have enjoyed varying popularity for giving comfort to individuals with obstructed upper respiratory passages. Such procedures usually require the patient to close off the posterior nasal space by elevating the soft palate and uvula against the posterior pharyngeal wall. However, with the subject supine and head extended 20 degrees or more (Fig. 1), fluid cannot readily pass into the larynx and trachea, despite the position of the soft palate and uvula, and in our experience the method is innocuous. An apparatus utilizing such a procedure was devised and in numerous trials has proved quite satisfactory.

The equipment is relatively simple, consisting of a sterile apparatus, a frame, and a source of negative pressure.

The sterile apparatus (Fig. 2, *A* and *C*) consists of two L-shaped glass tubes with 4 by 4 inch legs (*F*) and 2¼ by 4 inch legs (*X*), inserted into two openings of a three-hole No. 5 black rubber stopper (*R*), with the third opening containing a 6½ inch long glass tube (*A*) connected at its upper end by a 1½ inch flexible tube (*AB*) to a three-way metal stopcock (*SC*). A 13½ inch long flexible tube (*B*) connects the outlet of the stopcock with a nasal tip (*T-1*) by means of a 3½ inch glass tube (*C*). A similar nasal tip (*T-2*) and glass tube (*D*) is attached to a 9 inch long flexible tube (*E*) connecting with the end of the left lateral L-shaped tube (*F*). Nasal tips (*T-1*) have been made in three sizes from red rubber serum bottle stoppers by grinding down the sides.

All glass tubing is 7 mm. diameter Pyrex, annealed after bending. Flexible tubing may be of rubber or plastic but should be resistant to autoclaving and moderate negative pressure.

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In use, the set (wrapped as a unit and sterilized by autoclaving) is unwrapped and inserted by the rubber stopper (*R*) into a 100 ml. round-bottomed Pyrex centrifuge tube (or equivalent) (*CF*) containing 50 ml. of sterile nasal washing menstruum. A sterile 30 ml. syringe (preferably Luer Lock) (*S*) is attached to the stopcock.

The plywood frame (Fig. 2, *B*) for holding together and protecting the apparatus has a gun-shaped pattern. A ¾ inch thick plywood block (*L*) with a 1⅛ inch diameter hemispheric depression and a rectangular opening (*M*) 1⅛ by 5½ inches support the centrifuge tube. A ⅞ by 4¾ inch rectangular opening (*N*) holds the barrel of the 30 ml. syringe. Three small-

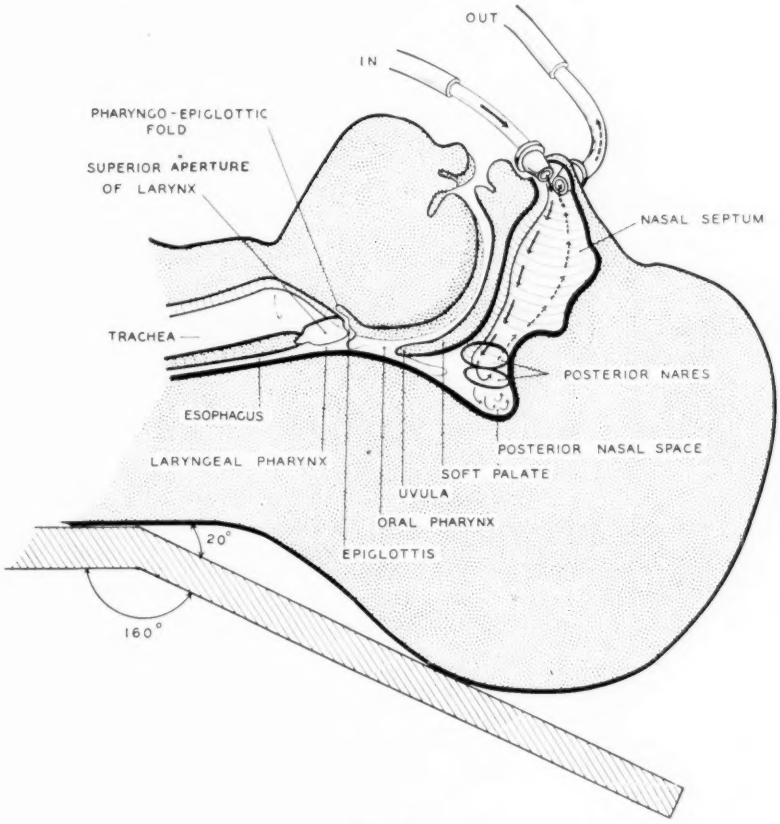
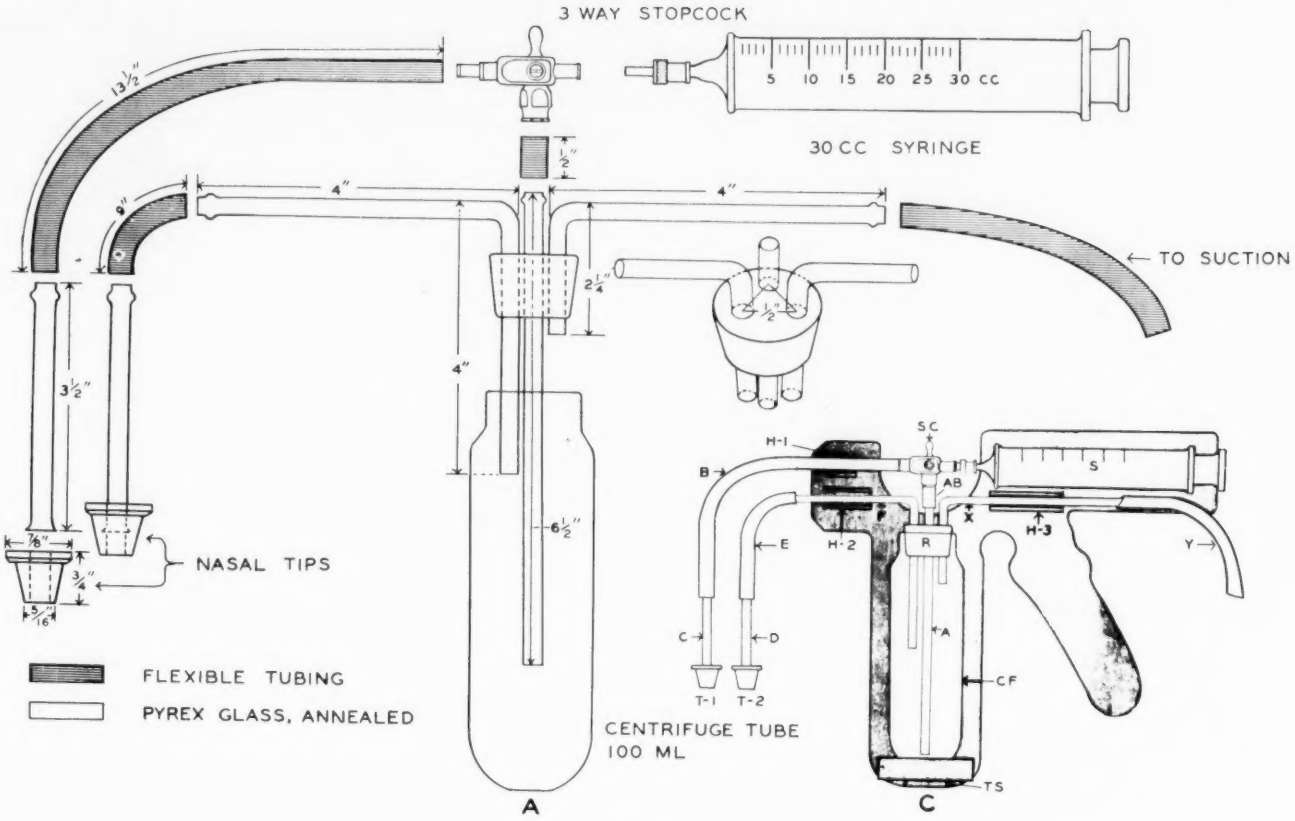


Fig. 1.

grooved pieces of wood (*H-1*, *H-2*, and *H-3*) support the tubing. They are secured to the frame by one screw apiece, which allows them to be turned to fit the tubing in the best possible manner. Attached to the base of the frame is a 1¼ inch diameter by ¼ inch thick threaded brass tripod socket (Fig. 2, *C*, *TS*). A sturdy camera tripod with telescoping legs and a good pan head is of value in supporting the apparatus at precisely the optimum position beside the donor's nose. Rubber bands conveniently secure the set to the frame, so that various-sized syringes and types of tubes containing nasal washing menstruum can be easily interchanged.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5 10 15 20 25 30 CC
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⅞"
¾"
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NASAL TIPS
FLEXIBLE TUBING
PYREX GLASS, ANNEALED
CENTRIFUGE TUBE
100 ML
A
H-1
SC
B
AB
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X
H-3
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Fig. 2.

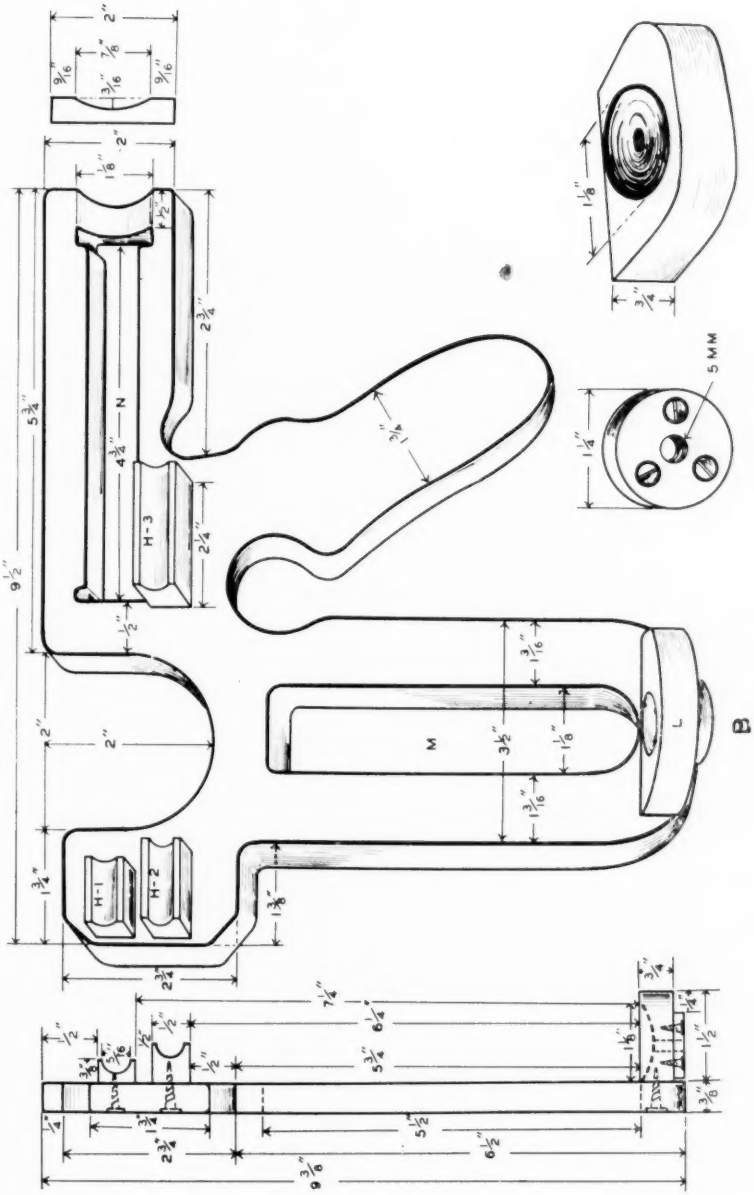

Fig. 2 (Cont'd).

A source of suction has been conveniently supplied by a water pump with adapters for various-sized water faucets. The optimum negative pressure is from 5 to 7 mm. of mercury.

TECHNIQUE OF OPERATION

The equipment is assembled as shown in Fig. 2, *C*. The sterile apparatus is unwrapped and the rubber stopper (*R*) inserted into a tube (*CF*) containing sterile nasal washing menstruum. A sterile 30 ml. syringe (*S*) is attached to the stopcock. Rubber bands are used to mount the assembly on the plywood frame.

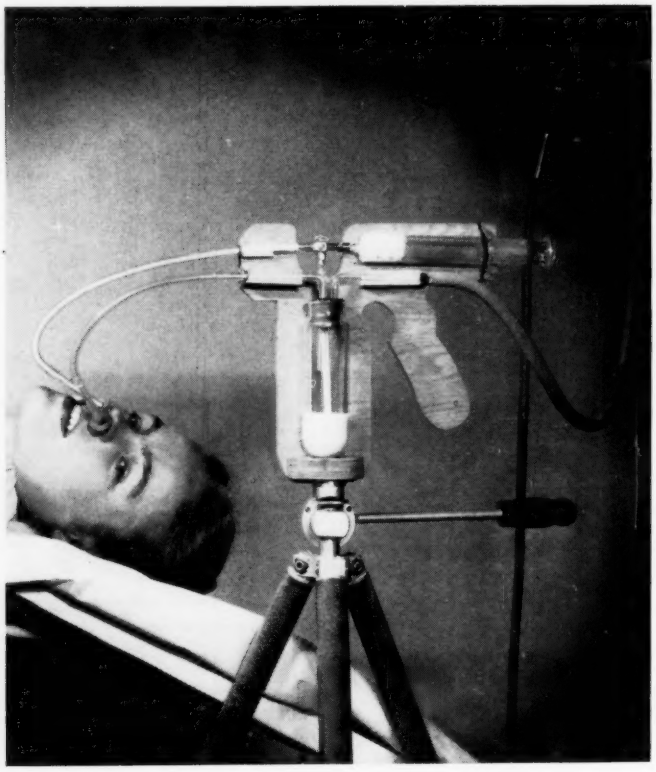

Fig. 3.

The suction tube (*Y*) is connected to the source of negative pressure. This is adequately tested by manually clamping tube *E* with the stopcock lever down (Fig. 4, *B*, position *a*). At the proper negative pressure, the 50 ml. menstruum begins to froth violently without, however, climbing into the L-shaped tube (*X*) connecting with suction.

The donor is requested to lie on the back with the head extended 20 degrees or more below the horizontal plane of the shoulders and body (Figs. 1 and 3). With the donor's head rotated to the side of the apparatus, nasal washing fluid can run out the side of the mouth into a sterile beaker should

the suction fail and the patient be unable to close off the posterior nasal recess by the soft palate and uvula. The nasal tips (Fig. 2, *C*, *T-1* and *T-2*) are gently but snugly inserted into the external nares and the donor instructed to breathe through the mouth. Usually the nasal tips must be kept in place by the operator, the donor, or an assistant.

Nasal washing is obtained in the following manner (Fig. 4):

1. With the stopcock lever up (Fig. 4, *A*, position *c*), the donor can usually feel the negative pressure. He is instructed not to swallow the menstruum.

2. With stopcock lever down (Fig. 4, *B*, position *a*), 30 ml. of nasal washing menstruum is drawn into the syringe.

3. Stopcock lever is turned to horizontal (Fig. 4, *C* and *D*, position *b*) and the material in the syringe is slowly emptied into the upper nostril, the posterior nasal recess of the nasopharynx, and through the lower nostril back into the apparatus (Fig. 1 and Fig. 4, *C* and *D*). When the syringe is free and the suction working well, the syringe will empty itself at the proper rate. Usually, however, the syringe must be manually operated. If the nasal washing menstruum is pushed through too rapidly (particularly if the suction is not adequate), fluid may overflow at the nasal tips, perhaps flow into the oral pharynx (where the patient may swallow it or expectorate it into a sterile beaker), or flow into the nasolacrimal duct to the eye.

Steps 2 and 3 are repeated as many times as desired. Utilizing this procedure, it is not difficult to circulate rather quickly 30 ml. amounts of menstruum through the nasal passages eight to ten times.

4. When the nasal passages have been sufficiently washed, the stopcock lever is turned up (Fig. 4, *E*, position *c*) so that the negative pressure cleans out both nostrils. The donor is instructed to inhale, sit up, and expectorate the material from the throat into the sterile beaker immediately after the nasal tips are removed. Usually, the expectorated material is only mucus showing no trace of the nasal washing menstruum. Often, however, the sinuses have been irrigated by the the washing procedure and a few milliliters more washing can be collected by having the donor flex the head and blow one side of the nose and then the other into the sterile beaker at intervals for two to five minutes.

The apparatus may be tipped slightly, if necessary, to capture the last few drops of mucus in the tubing. Suction is discontinued. The apparatus is disconnected from the tube containing the washing by pulling out the rubber stopper (Fig. 2, *C*, *R*). The material collected in the beaker may be poured into the tube if desired. The container (Fig. 2, *C*, *CF*) is closed by inserting a sterile No. 6 rubber stopper. Spraying with a vasoconstrictor will maintain the patient's comfort for a number of hours. This has not been necessary when sterile skim milk was used as the nasal washing menstruum.

Sterile skim milk has been the menstruum used for the routine nasal washings because many rickettsial agents survive longer and withstand freezing and drying better when protected by milk.[1, 2] Since a quantity of mucus has

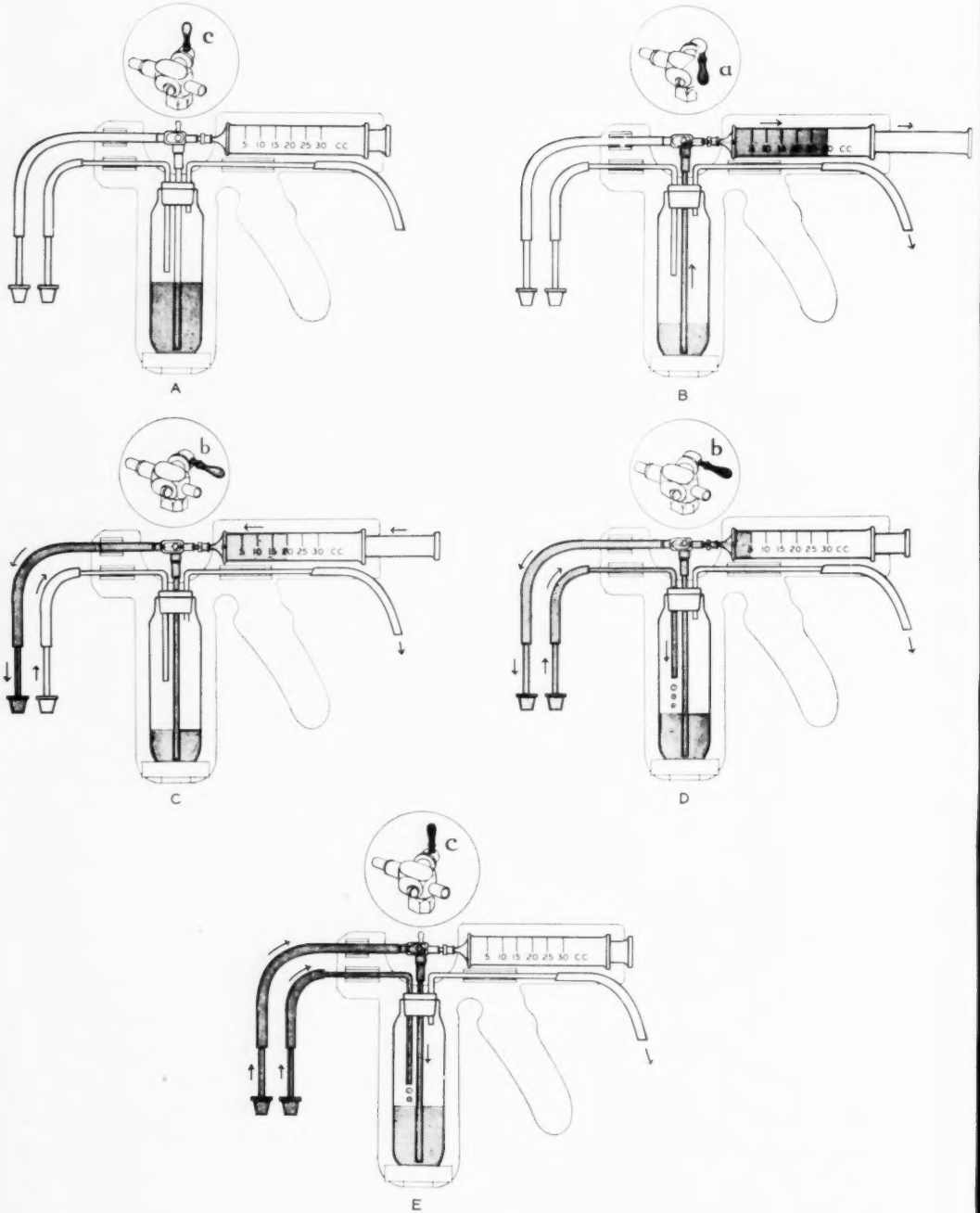

Fig. 4.

been present in most washings, homogenization in a Waring blendor or by hand has been routine. After centrifuging (to remove cellular debris), quick freezing, and storing at -50° C., washings are used for the inoculation of embryonated eggs, human volunteers, and various laboratory animals.

DISCUSSION AND SUMMARY

An apparatus and method for obtaining nasal washings is described. The apparatus and the method of its use have proved quite satisfactory during numerous trials. No complications have resulted from obtaining nasal washings in this manner.

Advantages of such a procedure are as follows:

1. It obtains washings from the nasal passages without serious contamination from oral material.

2. Washings reach areas in the nasal passages (such as sinuses and nasolacrimal ducts) not usually reached by previous methods.

3. Friable epithelial cells and cellular debris in nasal passages are collected more effectively by recirculating the washing material eight to ten times. The etiologic agents sought may be present in this cellular portion.

4. Washings are easily and efficiently collected.

5. Procedure is not as unpleasant as previous methods, and donors report a feeling of well-being and "clearheadedness" following the washing.

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A NEW RAPID TECHNIQUE FOR Rh TYPING

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IT IS being recognized increasingly that human isoimmunization to the Rh factors is a common occurrence. Whereas about only one in twenty-five Rh-negative women who bear Rh-positive infants ever develop Rh antibodies, Diamond[1] and Wiener and Sonn-Gordon[2] were able to sensitize a very large percentage of Rh-negative volunteers by injections of Rh-positive blood. Diamond,[1] Ross,[3] and Hattersley[4] likewise found Rh antibodies in the serum of about 50 per cent of previously transfused Rh-negative patients, demonstrating that sensitization by transfusion is not, as it has been considered for so long, a relatively rare occurrence. The danger to future pregnancies of such accidental sensitization in women is well established, and the fact is becoming recognized that transfusion reactions due to Rh sensitization occur not infrequently. Consequently, increasing numbers of hospital laboratories are establishing Rh typing as a routine pretransfusion procedure.

There are three important difficulties which are encountered when such a program is introduced: (1) Adequate supplies of reliable and potent Rh typing serum are not always available; (2) Rh typing techniques require skill which many laboratory technicians lack; (3) Rh typing requires time which, when blood is urgently needed, is at a premium.

The first of these difficulties is now largely solved by the increasing use of the hyperimmune or "blocking" antibody, which was first shown by Diamond and Abelson[5] to be capable, under certain conditions, of agglutinating Rh-positive cells. Sera containing such antibodies are of common occurrence, particularly among individuals sensitized by transfusions, and may reach extremely high titers. They have the further advantage of being more stable than saline agglutinins and, with the techniques suggested by Wiener[6] and Diamond and Denton,[7] are equally easy to use.

On the other hand no method of Rh typing which is at once uniformly easy to read and very rapid has yet been reported. The tube techniques, while perhaps the simplest to interpret, have the drawback of requiring incubation. The slide technique of Diamond and Abelson,[5] while it gives Rh typing in five minutes or less, occasionally gives confusing or equivocal results, and the somewhat less rapid capillary method of Chown[8] requires a skill of handling which makes it impractical for many smaller laboratories.

While attempting to solve these problems as they existed in this laboratory, it was discovered that when Rh-positive cells are mixed with a high titer hyper-

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immune Rh antiserum and immediately centrifuged at high speed, there is gross agglutination which is not readily broken up. On the basis of this observation, the following technique was established:

> A potent hyperimmune serum (with alpha and beta antibodies neutralized) is diluted with several volumes of 20 to 30 per cent bovine albumin. One drop of this mixture is placed in a small agglutination tube. An applicator stick is thrust into the clotted or oxalated whole blood to be tested and enough cells carried over to the agglutination tube to make a moderately heavy suspension in the drop of serum.* (If preferred, a small drop of a suspension of cells in their own serum or in saline may be used, taking care that the concentration of albumin in the final mixture does not fall below 15 per cent.) The tube

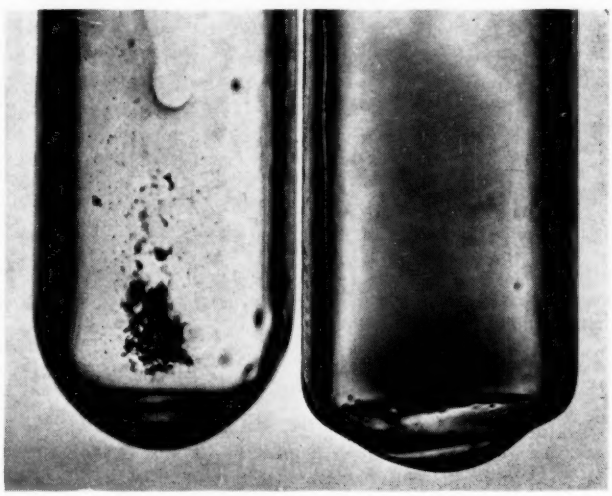

Fig. 1.—The Rh test, ready to read. Tube on the left shows gross agglutination of Rh-positive cells. Tube on right shows smooth suspension of Rh-negative cells. (Courtesy Letterman General Hospital Photographic Laboratory.)

> is shaken to insure mixing and without preliminary incubation is centrifuged at high speed for two minutes. It is then shaken gently and the sediment examined for agglutination. Rh-positive cells show gross clumping which is not readily broken up. Rh-negative cells remain perfectly smooth, without rouleaux (Fig. 1).

Since this technique was introduced into our laboratory, where it has been used in parallel with one of the standard methods, it has proved entirely reliable in more than 1,000 tests. It has been found so easy to handle that even our least skilled technicians have had no trouble with it, and its great speed has on several occasions proved invaluable to us.

Certain precautions are necessary in choosing a suitable serum for use with this technique. Sera with an initial titer in albumin of less than 1,000 are

*This portion of the technique was suggested by Miss Evelyn Tennis of the Community Blood Bank, San Mateo, Calif.

seldom satisfactory, and the final titer should not be below 100. Even among sera of adequate titer some will be found which are insufficiently avid to give satisfactory results, and these sera must be eliminated by trial and error. Care should likewise be taken to standardize the serum as to specificity, for, as shown by Diamond[1] and Hattersley,[4] hyperimmune antibodies of Rh′ (C of Fisher) and Rh″ (E) specificity not infrequently occur, mixed with the more common Rh_0 (D) antibody.

It is felt that if these few precautions are observed this rapid technique should prove of considerable value, particularly in laboratories such as ours where speed and ease of Rh typing are at a premium, and should render it increasingly practical to determine routinely the Rh type of all transfusion cases.

SUMMARY

A new technique of Rh typing is described. It utilizes the readily available Rh "blocking" serum and has the advantage of speed and of ease of reading. It has proved entirely reliable in a series of over 1,000 bloods.

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THE PHOTOELECTRIC DETERMINATION OF MAGNESIUM IN BODY FLUIDS

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DURING the course of investigations in connection with magnesium studies, it was found that the principal methods for the quantitative estimation of magnesium in biologic fluids were tedious and laborious. These methods required the precipitation of magnesium as the oxyquinolate[1] or as the phosphate,[2] and the preliminary removal of calcium by precipitation as the oxalate was also necessary.

Kolthoff[3] observed in 1927 that magnesium ions in the presence of titan yellow (thiazol yellow, Clayton yellow), produced a red color in an alkaline solution. Since then several workers have described methods for the determination of magnesium which are based upon this principle. Methods have been described for the analysis of the magnesium content of fertilizers and soil extracts by Gillam,[4] of plant tissues by Lindner,[5] and of water by Ludwig and Johnson.[6]

The procedure presented in this paper offers several advantages over the existing methods and also is adapted to the analysis of erythrocytes and of urine.† The blood, serum, or plasma proteins are precipitated by means of tungstic acid. An aqueous solution of titan yellow is added to the water-clear filtrate, and sodium hydroxide is added to develop the red magnesium hydroxide-titan yellow complex. Hydroxylamine hydrochloride is used to stabilize the color lake.

Reagents.—

10 per cent solution of sodium tungstate dihydrate.

0.67 N sulfuric acid. (This and the tungstate solution must be free of magnesium.)

6.0 per cent sodium hydroxide solution.

Approximately 0.1 N sodium hydroxide.

2 per cent hydroxylamine hydrochloride solution.

4 per cent hydroxylamine hydrochloride solution.

0.075 per cent titan yellow solution. Prepared by dissolving 150 mg. of titan yellow in 200 ml. of water. This solution should be protected from light. Thirty minutes in direct sunlight will cause enough destruction to render the solution unreliable. It should be refrigerated and a fresh solution prepared about every ten days.

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†The major portion of the work presented in this paper had been completed when a paper by Garner[7] appeared describing a method for either serum or plasma. While the method by Garner is simple and rapid, it differs in several respects from the method reported in this paper.

Methyl red indicator. Prepared by mixing 50 mg. of methyl red in 50 ml. of 95 per cent ethanol.

Standard stock solution of magnesium. 10.131 Gm. of magnesium sulfate heptahydrate are dissolved in distilled water, 0.5 ml. chloroform added, and the volume made to one liter with distilled water. One milliliter of this solution contains 1 mg. of magnesium. This is then diluted to the proper concentrations.

Procedure for Serum and Plasma.—To one volume of serum in a small flask add two volumes of water, one volume of 10 per cent sodium tungstate solution, and mix. To this solution add one volume of the 0.67 N sulfuric acid. Mix thoroughly and filter, using a Whatman No. 41 filter paper. The filtrate should be water clear.

An aliquot of 3 to 5 ml. of the filtrate is transferred to a 10 ml. volumetric flask and one drop of the 0.01 per cent methyl red indicator is added. Carefully neutralize the solution just to the yellow color of the indicator. Then add successively 1 ml. of the 2 per cent hydroxylamine hydrochloride, 1 ml. of the 0.075 per cent titan yellow solution, and 2 ml. of the 6.0 per cent sodium hydroxide. Add immediately enough water to bring the solution to volume and mix thoroughly. Bring the temperature of the solution to approximately 25° C. and read the transmission (or absorption) colorimetrically or spectrophotometrically at a wave length of 540 millimicrons. The blank is prepared by substituting distilled water for the filtrate and is treated exactly as the unknown.

Procedure for Whole Blood.—One volume of oxalated blood is laked with seven volumes of water. To this solution add one volume of 0.67 N sulfuric acid followed by one volume of the 10 per cent sodium tungstate. Mix thoroughly and filter. Generally a more complete deproteinization is obtained by adding the acid before the sodium tungstate.

In the case of bovine blood or other blood where the magnesium content of the erythrocytes is approximately the same as that of the plasma, it may be convenient to prepare a 1:5 filtrate instead of the 1:10 filtrate by laking with only two volumes of water. This gives a filtrate with a higher magnesium content and better precision may be obtained. In any case, blood oxalated with ammonium oxalate cannot be used since the ammonium ion interferes with the determination. Potassium and sodium oxalates have been used without difficulty. After the filtrate is obtained, the procedure for the magnesium determination is the same as outlined for serum or plasma filtrate.

Procedure for Erythrocytes.—Erythrocytes may be obtained by centrifuging whole blood for thirty minutes at about 3,500 revolutions per minute and by taking up the erythrocytes by means of an Ostwald-Folin pipette. Add one volume of erythrocytes to five volumes of distilled water and allow the hemoglobin lake to form. Then add two volumes of 0.67 N sulfuric acid followed by two volumes of the 10 per cent sodium tungstate solution. Mix thoroughly and filter. After the filtrate is obtained, the procedure as outlined for serum or plasma filtrate is followed. It is advisable, however, to use a smaller

aliquot (2 to 3 ml.) if the blood is that of swine, rabbits, or any other species where the magnesium content of the erythrocytes may be high.

Procedure for Urine.—Since the color of urine varies with the individual sample, thereby causing some error, a like aliquot of the urine is treated with all reagents except the titan yellow reagent. The amount of optical density contributed by the colored urine constituents may then be determined and the results corrected accordingly.

To each of two 25 ml. volumetric flasks containing exactly 5 ml. of the 4 per cent hydroxylamine hydrochloride solution add enough urine to contain 0.2 mg. to 1.0 mg. of magnesium. To the first flask only, add 3 ml. of the 0.075 per cent titan yellow solution. Enough distilled water is then added to bring the solution to a volume of approximately 19 milliliters. To both solutions add 5 ml. of the 6.0 per cent sodium hydroxide solution. Make to volume with distilled water, adjust the temperature to about 25° C., and read each solution in the colorimeter or spectrophotometer at 540 millimicrons. Prepare the respective blanks (one with and one without the titan yellow) in exactly the same manner, with the urine being left out.

The amount of urine required for the determination of magnesium varies considerably, depending on the volume of urine excreted and the dietary intake of magnesium. With experience the analyst can easily determine the approximate size of sample required. It is desirable, though not essential, to dilute the urine sample prior to analysis. This allows greater precision, and the sediment which occurs in some samples of urine usually goes into solution when the urine is diluted. In the case of horse urine, when certain organic sediments remain, it may be necessary to neutralize and filter even after dilution.

It is generally unnecessary to precipitate the proteins which may be present in the urine unless the urine is discolored by blood. If deproteinization is necessary, to eight parts of urine add one part of the 10 per cent sodium tungstate solution and one volume of the 0.67 N sulfuric acid. Mix thoroughly and filter.

Calibration Curve.—The diluted magnesium sulfate standard solution is used in preparing the calibration curve. A series of 10 ml. volumetric flasks is set up containing the following amounts of magnesium: 0.0 (blank), 10, 20, 30, 40, 50, and 60 micrograms. The standard is then treated exactly as described for serum or plasma filtrate. The density or transmittance, depending upon which reading is taken, is plotted along the ordinate and the amounts of magnesium along the abscissa of the graph.

It may be more convenient to establish a constant for the instrument and calculate the unknown concentration from the following formula:

$$\text{Log } \frac{Io}{I} = KC1$$

where

C = The concentration

K = Constant for the instrument (Determined with the standards where C is known)

l = Length of the cell in centimeters

Io = The intensity of the incident light (Where the galvanometer is set at 100, log Io = 2)

I = The intensity of the transmitted light (Galvanometer reading of the unknown samples)

Transmittance measurements made with the Evelyn photoelectric colorimeter (Fig. 1) showed that Beer's law holds precisely over the range of 0.0 to 50 μg. of magnesium in 10 ml. of solution. No deviation from the logarithmic straight line of the curve at the lower magnesium concentration occurred when the titan yellow and the hydroxylamine hydrochloride were of adequate strength. The curve obtained fifteen minutes after adding the sodium hydroxide when no hydroxylamine hydrochloride was added is shown as curve *A* in

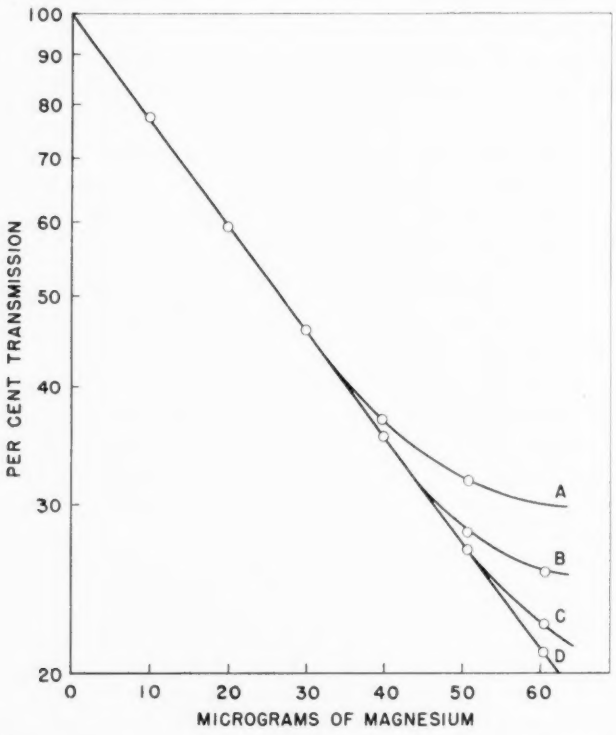

Fig. 1.—Calibration curves with Evelyn photoelectric colorimeter showing effects of various amounts of hydroxylamine hydrochloride and titan yellow. *A*, 1 ml. 0.05 per cent titan yellow and no $NH_2OH \cdot HCl$; *B*, 1 ml. 1 per cent $NH_2OH \cdot HCl$ and 1 ml. 0.05 per cent titan yellow; *C*, 1 ml. 2 per cent $NH_2OH \cdot HCl$ and 1 ml. 0.05 per cent titan yellow; and *D*, 1 ml. 2 per cent $NH_2OH \cdot HCl$ and 1 ml. 0.075 per cent titan yellow.

Fig. 1. Where no hydroxylamine hydrochloride was added, the color was less stable, as indicated by a greater deviation from the straight line which became more pronounced the longer the solutions were allowed to stand. Suboptimum levels of hydroxylamine hydrochloride and titan yellow show varying degrees of deviation (curves *B* and *C*).

Wave Length.—The optimum wave length reported by Ludwig and Johnson[6] was 525 millimicrons. It was found in the present work that the sensi-

tivity of measurement as determined with the Coleman Junior Spectrophotometer is greatest at 540 millimicrons. A filter with the maximum transmittance at 540 mμ was used in the routine determinations with the Evelyn colorimeter.

Recovery Experiments.—Serum was obtained from various species by allowing freshly drawn blood to clot and storing it in the refrigerator for a few hours. Each sample of serum was analyzed for magnesium according to the technique described. Known amounts (10 to 20 μg per milliliter) of magnesium, as magnesium sulfate, were then added to the serum and analyses were run to measure the recovery of the added magnesium. Data were obtained from twenty-four determinations on twelve different serum samples. The mean recovery of the added magnesium was 99.84 per cent, with a standard deviation of ± 1.44 per cent. Whole blood was oxalated by means of potassium or sodium oxalate. The procedure used in the recovery experiments was the same as that used with serum, except that 20 to 40 μg of magnesium were added. The mean recovery of thirty-seven determinations on twelve different blood samples from different species was 99.47 per cent, with a standard deviation of ± 1.32 per cent.

Urine obtained from male adults was used in the recovery experiments. The mean recovery for ten determinations of added magnesium to ten samples was 99.65 per cent, with a standard deviation of ± 2.22 per cent.

TABLE I. RECOVERY OF MAGNESIUM ADDED TO SAMPLES OF SERUM, WHOLE BLOOD, AND URINE

	SERUM	WHOLE BLOOD	URINE
Number of samples	12	12	10
Number of determinations	24	37	10
Mean per cent magnesium recovered	99.84	99.47	99.65
Standard deviation	±1.44	±1.32	±2.22

In all recovery experiments there was no constant error in the method since the per cent recovery was neither consistently above nor below the expected value.

Ten samples of serum were independently analyzed by the described method on different days by two different analysts using two entirely different sets of reagents. The correlation coefficient between the magnesium values obtained by the two different individuals was + 0.985. Also in analyses of ten samples of whole blood, different amounts of filtrate (3 to 5 ml.) were used and a correlation coefficient of + 0.961 was obtained. There was no constant error, for the values obtained using smaller aliquots were neither consistently above nor below the values obtained using larger aliquots.

Remarks.—Calcium and phosphate ions interfere, but fortunately these substances have not been found to be present as blood components in concentrations that appreciably influence the results. The calcium concentration, as reported by Gillam,[4] in order to interfere must be at least 500 μg per milliliter in the solution that is read photometrically. The phosphorous concentration must be kept below 100 μg per milliliter since it interferes by buffering the solution and lowering the hydroxyl ion concentration. If in urine, for

example the urine of a calf receiving only a milk diet for some time, the phosphorus-magnesium ratio is wider than 200:1, the method described in this paper is limited to a semiquantitative determination. If such urine is to be analyzed, the technique for soil extracts described by Gillam[4] is recommended.

The hydroxyl ion concentration must be carefully controlled. The higher the hydroxyl ion concentration, the greater the density of the solution. Acid, base, or buffering solutions (hydroxylamine hydrochloride solution) must be added carefully so as to insure the same amounts in the unknown sample as in the blank. In the method described by Garner[7] the alkalinity of the unknown was greater than that of the blank, for the method did not take into account the trichloracetic acid removed from the solution by the precipitation of the proteins. The values were higher than those obtained when the trichloracetic acid had been removed.

The temperature of the solution affects the optical density of the solution and must be controlled. The specific temperature is not of importance but the unknown sample should be read at approximately the same temperature as the standards. It is more convenient to adjust to room temperature; therefore the temperature selected was 25° C.

TABLE II. THE MAGNESIUM CONTENT OF WHOLE BLOOD AND PLASMA

	WHOLE BLOOD			PLASMA OR SERUM		
SPECIES	NUMBER OF ANIMALS	AVERAGE (MG./100 ML.)	RANGE* (MG./100 ML.)	NUMBER OF ANIMALS	AVERAGE (MG./100 ML.)	RANGE* (MG./100 ML.)
Human being	6	3.82	3.58-4.50	6	2.14	2.02-2.22
Sheep	6	2.79	2.61-2.92	6	2.27	2.02-2.47
Rabbit	7	5.23	4.72-5.73	6	2.45	2.05-2.97
Pig	6	5.26	5.03-5.60	8†	2.46	2.20-2.84
Cattle	6	2.04	1.95-2.09	45†	2.13	1.23-2.75

*Range refers to observed concentrations.
†Serum.

Magnesium Content of Blood.—The magnesium content of whole blood and plasma or serum from five different species has been determined by the method reported here. The bloods were collected at random from healthy individuals and animals. The results (Table II) show that the concentration of magnesium in the plasma or serum varies little among the different species. There was considerable variation in the magnesium content of whole blood. The magnesium content of the whole blood of the rabbit and pig was around 5.24 mg. per 100 ml., whereas for cattle and sheep the values were less than 3.0 mg. per 100 milliliters. In the human being, sheep, rabbit, and pig the concentration of magnesium in the whole blood is greater than that in the plasma or cells. These observations are in accord with reports on the distribution of magnesium in the blood of cattle[8] and human beings.[9]

SUMMARY

A method has been described for the photoelectric or spectrophotometric determination of the magnesium content of serum, plasma, whole blood, erythro-

cytes, and urine. The blood proteins are precipitated by means of tungstic acid, and the magnesium is measured by the amount of the red magnesium hydroxide-titan yellow complex produced. The mean recoveries of added magnesium ranged from 99.5 to 99.8 per cent with whole blood, serum, and urine.

Data are presented on the magnesium content of whole blood and plasma for the human being, rabbit, pig, sheep, and cattle.

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RAPID TITRIMETRIC MICROMETHOD FOR THE DETERMINATION OF NONPROTEIN NITROGEN

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IT IS possible to determine the nonprotein nitrogen in whole blood, serum, or plasma within a few minutes with the accuracy of the Kjeldahl method[1] but without the troublesome digestion and distillation. An alkaline hypobromite-boric solution is added to a protein-free filtrate and the excess of bromine is titrated iodometrically.[2, 3] Boric acid has been added to the deaminating solution to prevent any interaction between the bromine and the glucose present in protein-free filtrates, since even large amounts of glucose are bound by boric acid. Not only urea and ammonia, but all physiologic amino compounds are included, the latter being deaminated simultaneously. Even amino acid mixtures obtained by protein digestion with trypsin in the preparation of broth give identical results using this and the Kjeldahl method. Indole compounds which contain nitrogen in their ring system do not affect the result since normal blood contains only traces of these compounds.[4] Even in uremia, indole compounds do not interfere since their nitrogen contribution does not rise above 0.4 to 0.5 mg. per cent.

METHOD

Reagents.—

A. *Deproteinizing Solutions:*

1. Dissolve 20 Gm. sodium sulfate anhydrous in water, add 69 ml. of $\frac{2}{3}$ N sulfuric acid and fill the flask with water to the 1,000 ml. mark.

2. Dissolve 5 Gm. sodium sulfate and 8.3 Gm. phosphomolybdic acid in about 200 ml. water; add 20 ml. of 5 N sodium hydroxide solution and boil gently over a burner for thirty minutes. Cool; add 10.6 ml. of concentrated sulfuric acid, then 10 Gm. cadmium sulfate, and make up to final volume of 1,000 milliliters.

Cadmium sulfate has been added to the deproteinizing solution in order to precipitate compounds which would evolve hydrogen sulfide and which would therefore use up relatively large amounts of bromine. However, cadmium sulfate precipitates all surfur compounds.

B. *Preparation of Deaminating Reagent:*

1. Boric acid solution. Dissolve 84.5 Gm. boric acid and 15.6 Gm. sodium hydroxide in about 500 ml. water and boil for thirty minutes. Cool and make up to one liter.

2. Sodium fluoride solution saturated.

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3. Sodium hydroxide, 27 per cent. Made by dissolving 27 Gm. in 100 ml. of water.

4. Bromine solution. Dissolve 20 Gm. potassium bromide in about 50 ml. water; add 8 Gm. (2.5 ml.) pure bromine; mix until bromine dissolves and make up to one liter.

If it is more convenient, the bromine solution may be made as follows: Dissolve 32 Gm. potassium bromide and 2.8 Gm. potassium bromate ($KBrO_3$) in some water in a liter volumetric flask; add 100 ml. of normal sulfuric acid and after waiting for thirty minutes, fill up to the 1,000 ml. mark with water.

5. Boric acid-fluoride mixture. Mix 5 parts boric acid solution (1) with 3 parts sodium fluoride (2) and 1 part 27 per cent sodium hydroxide (3) [for example, to 250 ml. (1) add 150 ml. (2) and 50 ml. (3)]. This mixture keeps well.

6. Deaminating reagent. Just before use add 1 part of bromine solution to 9 parts of boric acid-fluoride mixture.

C. *Titration Reagents:*

1. Sodium thiosulfate N/200.
2. Potassium iodide crystals.
3. Starch solution 0.25 per cent.
4. Hydrochloric acid 18 per cent (Approximately 5 N).

Procedure.—Pipette 2.9 ml. deproteinizing solution (A1) into short, stoppered test tubes. With a capillary pipette add 0.1 ml. blood directly from finger and lake by repeatedly drawing fluid in and out of the pipette. Add 1 ml. of deproteinizing solution (A2). Mix, let stand five minutes, and then centrifuge or filter (0.2 ml. blood may be used with 5.8 ml. deproteinizing solution, and 2 ml. of phosphomolybdic acid).

Into two wide test tubes or flasks, pipette 3 ml. of clear filtrate and 5 ml. of deaminating solution. Mix and, after two minutes, add a few crystals of potassium iodide and 2 to 3 ml. of hydrochloric acid to each sample and titrate with N/200 sodium thiosulfate until yellow; finally, add a few drops of starch solution and titrate until disappearance of the blue color.

As a blank, mix 3 ml. of deproteinizing solution (1) with 1 ml. of deproteinizing solution (2); take 3 ml. of the mixture to add to 5 ml. of deaminating solution and titrate with the thiosulfate solution.

Calculation.—Since $2NH_3 + 6Br = 6HBr + N_2$, one atom of N is equivalent to 3 atoms Br. Thus 1 ml. N/200 thiosulfate is equivalent to

$$\frac{14 \text{ (atomic weight N)}}{3 \times 200} = 0.0233 \text{ mg.} \quad \frac{100}{0.075 \text{ (ml. sample used)}} \times 0.0233 = 31.1.$$

From the milliliters of thiosulfate used for the blood sample, subtract the milliliters used for the reagent blank, and obtain the milliliters actually used by the blood sample. Multiply this by the factor 31.1 to obtain the nitrogen in milligrams per cent.

ALTERNATE METHOD

Reagents.—

A. *Deproteinizing Solution* (Modified Abrahamson's Reagent)[5]:

Dissolve 4.48 Gm. sodium tungstate, 2 Gm. sodium citrate and 6.4 Gm. sodium sulfate in about 500 ml. water; add 44.8 ml. of a normal sulfuric acid or 67.2 ml. 2/3 N sulfuric acid; then add 2 Gm. cadmium sulfate and fill volumetric flask to 1,000 milliliters.

Procedure.—Pipette 5.0 ml. deproteinizing solution A into a short test tube. Wash in 0.1 ml. finger-tip blood from capillary pipette. After five minutes, centrifuge or filter. Place 4 ml. of clear filtrate in another tube and continue with deamination and with the thiosulfate titration as described. As a blank, use 4 ml. of deproteinizing solution and 5 ml. of deaminating solution.

Calculation.—Subtract the number of milliliters of N/200 sodium thiosulfate used in the blank determination from the amount used by the blood sample, and multiply by 29.75 as a factor to obtain the amount of nitrogen in milligrams per cent, since 0.0784 ml. of blood is the amount of blood used in the determination.

SUMMARY

A rapid, accurate, and convenient titrimetric procedure has been described for the estimation of nonprotein nitrogen in serum, plasma, or whole blood. Since this method does not require digestion and distillation of the sample, many errors are avoided which are inherent in the longer procedure.

TABLE I. COMPARISON OF THE PRESENT METHOD WITH THE KJELDAHL METHOD FOR DETERMINATION OF NONPROTEIN NITROGEN

NUMBER	MICRO-KJELDAHL METHOD[1] (MG. PER 100 ML.)	PRESENT METHOD (MG. PER 100 ML.)
1	199	199
2	213	215
3	31.1	32.6
4	31.1	29.5
5	15.5	14.5
6	23.3	23.3
7	18.6	17.1
8	44.0	43.5

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FIBRIN APPEARANCE TIME

A Rotating Tube Method for Estimating the Clotting Time of the Blood

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THERE is great need for a test to reflect more reliably the fine alterations in the over-all picture of the coagulability of the blood than is possible by current methods. The results are influenced especially by (1) the constituents of the blood, (2) crude technical manipulation, and (3) uncertain criteria for fixing the time of clotting, the end point. Items 2 and 3 are amenable to control, and a technique which would make them more constant or precise would yield a more critical test than those in general use.

Considerable variation in the content, or activity, of one or more factors in the clotting process may occur without demonstrable alteration in the coagulation time, as it is commonly determined. This is well demonstrated in dicumarol- or salicylate-induced hypoprothrombinemia. It is possible, however, that a more sensitive test would reflect regularly the inhibition of the clotting process in the presence of reduced activity of a single component such as prothrombin.

In the manipulation of the blood specimen there are two major factors which alter the coagulation time: (1) the nature of the container and (2) trauma to the formed elements of the blood.

Nature of the Container.—When kept in "nonwettable" vessels, blood may clot more slowly. This can be controlled by using the same type of vessels of approximately equal size and smoothness at all times. Inconstancy in the relationship of glass contact surface to total volume of blood will give considerable alteration in clotting time. Thus, by the Howell technique, using 21 mm. diameter tubes, the clotting time of normal blood may extend to sixty minutes; by the Lee-White method, using tubes of 8 mm. in diameter, the normal range for blood coagulation is from four to sixteen minutes; by the capillary tube methods, with tubing of less than 1 mm. in diameter, the normal clotting time is likewise highly variable. Nor can blood be poured indiscriminately against the glass surfaces of tubes without affecting adversely the reliability of the test. This can be demonstrated by comparing the Lee-White clotting time of a slightly tilted and of a fully tilted set of tubes. We have observed that not only the frequency and angle of tilting, but also the constancy of direction of tilting make a difference in the clotting time.

Trauma to Blood Constituents.—Blood which is forced under pressure through a small aperture, or is shaken or stirred, will clot promptly due to trauma to the formed elements, especially platelets, and to the liberation of the coagulating factors into the blood sample. To obviate this, it is essential that the blood be transferred consistently and gently.

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Lee-White Coagulation Test.—If a tube containing blood is tilted at regular intervals, the viscosity of the blood increases gradually until it forms a gelatinous mass which projects above the liquid portion when the position of the tube is changed. Later, the blood solidifies except for a small amount of liquid at the rim. Eventually, when the whole mass becomes solid enough to permit inversion of the tube without spilling, the end point is reached. Frequently the end point remains in doubt for several minutes, adding to the crudeness of the procedure. This difficulty is magnified in cases of disturbed clotting found in heparin medication and in blood dyscrasias.

FIBRIN APPEARANCE TIME

The following procedure minimizes the variables noted previously by providing (1) a nontraumatic manipulation, (2) constant agitation, and (3) a precise end point.

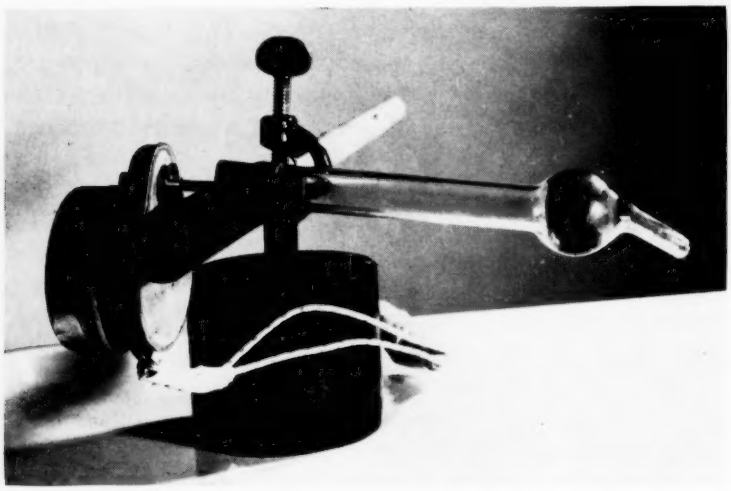

Fig. 1.—Photograph of apparatus showing tube attached to motor (see text).

In its liquid state, a small quantity of unmodified blood will remain, by virtue of its cohesive force, in a concave smooth container in the form of a large drop and will not attach itself to the surrounding surface. The instant blood commences to clot, fibrin forms and, because of its adhesiveness, clings to the container wall. This change is immediately detectable if the blood is contained in a transparent tube rotating at a slow and constant rate. Then, when fibrin forms it attaches to and climbs with the rising wall.

Apparatus (Fig. 1).—

1. A transparent tube of glass or plastic material about 11 mm. in diameter with a bulbous dilatation at one end from which there extends an eccentrically directed opening for the introduction of the blood sample.

2. A motor which rotates at a constant slow rate of about 1 revolution per minute.

3. An attachment to the shaft of the motor to hold the tube (Fig. 1).

Procedure.—A sample of blood is obtained by smooth venipuncture. At the instant of venipuncture the stop watch is started. One-half of one milliliter, about 8 drops, is immediately introduced into the bulbous end of the tube, which is attached to the shaft of the motor, and the motor is started. The blood forms into a large drop which remains in the well of the bulb. As soon as fibrin forms, the strands adhere in a growing mass to the surrounding bulb and climb with the rising wall. This appearance of a continuous mass of fibrin is the end point.

In one hundred estimations, made in eighty normal individuals, in which the rotating tube was subjected to the heat of a strong electric light, the time of fibrin appearance varied between 3.5 and 5 minutes. In thirty-one tests made in an equal number of cases at room temperature there was an average deviation of 33 seconds from a mean of 5 minutes and 18 seconds. We accept 4 to 6 minutes as the normal range at room temperature. In all normal cases in which blood was drawn gently after smooth venipuncture, the clotting time fell within the above range.*

A comparison was made of the clotting time of blood by the Lee-White method and the rotating tube method. Specimens of blood were obtained by "clean" venipuncture on twelve patients with a variety of chronic diseases. Each sample was divided instantly, appropriate quantities being used for the respective tests. The average results obtained are shown in Table I.

TABLE I

	AVERAGE TIME (MIN.:SEC.)	AVERAGE DEVIATION FROM MEAN (MIN.:SEC.)	% DEVIATION FROM AVERAGE
Rotating tube	5:15	0:39	12
Lee-White	10:38	2:36	24

From these data it can be seen that while the average clotting time by the Lee-White method is only twice as long as that by the rotating tube, the average deviation is four times as great.

The figures obtained are shown in Table II.

TABLE II

	ROTATING	LEE-WHITE
1	5:00	14
2	6:20	14
3	5:50	13
4	5:20	12½
5	6:15	8½
6	4:45	11
7	4:05	5½
8	4:10	8
9	5:25	7
10	5:00	8½
11	6:10	13
12	4:45	12.5

*If it is desired to make the test at body temperature, this can be accomplished by placing a strong electric bulb near the apparatus. The distance must be determined by trial and the apparatus kept at the optimum point for a time interval adequate to insure the desired temperature.

Patients being treated with dicumarol present good correlation between prolonged prothrombin time and fibrin appearance in the rotating tube (Fig. 2). Fibrin appearance time will not replace prothrombin time estimations in following dicumarol therapy, but it may extend the interval between essential prothrombin time estimations to about every five days. Fibrin appearance time is taken daily at the bedside. It has been found to become prolonged to about ten minutes at the dicumarol therapeutic level. It restores to normal more promptly than the prothrombin time. As soon as the fibrin appearance time becomes normal the prothrombin estimation is made again, and further dosage of dicumarol is determined from these prothrombin time values.

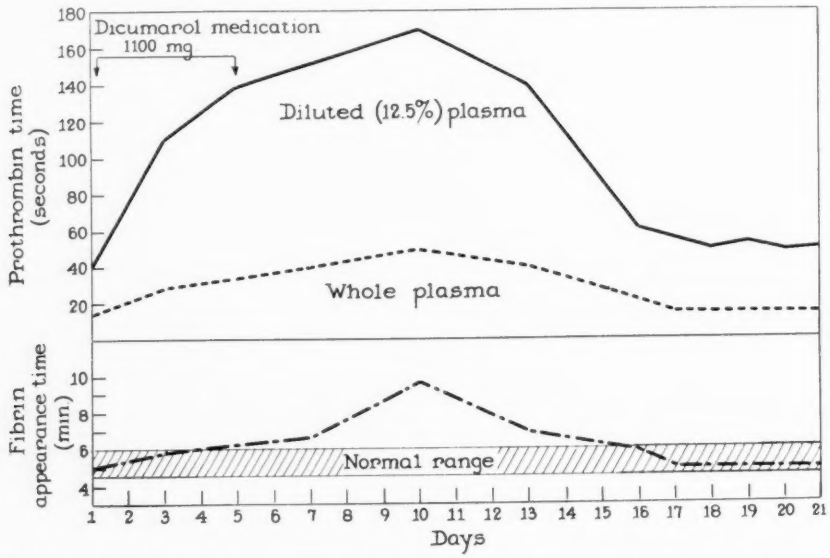

Fig. 2.—Chart illustrating extended fibrin appearance time in relation to prolonged prothrombin time of whole and diluted (12.5 per cent) plasma after 1,100 mg. of dicumarol.

The fibrin appearance method has a high correlation in heparin therapy as well. While the end point in the Lee-White method is vague and uncertain after heparin, the fibrin, appearance time method reveals a clear and unmistakable end point. We have found a good therapeutic level after heparin to be about twelve minutes.

In blood dyscrasias, characterized by extended coagulation time, the end points are likewise clear and the results reproducible. Thus we have found the fibrin appearance time to be 35 minutes in hemophilia. In thrombocytopenic purpura the time has been definitely, although only slightly, prolonged during the active bleeding phase of the disease. In the presence of clinical thrombosis in the acute stage the fibrin appearance time has been found to be normal or reduced.

SUMMARY

A method of precisely determining the over-all coagulability of unmodified blood by estimation of the time required for the appearance of fibrin is described. The test is made in a transparent tube rotating at a constant and slow rate.

It registers small increases in coagulation time, thus permitting a more critical estimation of alteration in blood coagulability.

It is applicable to the determination of the maintenance of therapeutic levels of heparin and/or dicumarol. Although it does not eliminate entirely the need for prothrombin time estimations after dicumarol, it may prolong considerably the intervals between essential prothrombin time estimations.

It may be possible to demonstrate acceleration of coagulation time by the new procedure.

A RAPID TEST FOR ALBUMIN AND SUGAR IN THE SAME MEASURED SAMPLE OF URINE

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TESTS to determine the presence of albumin and sugar in urine are ordinarily made on two different samples of the specimen. It would be advantageous to be able to make these two tests on the same measured sample of urine. The procedure described permits this to be done. Sulfosalicylic acid is the reagent used for albumin, and Benedict's solution, for sugar.

METHOD

Deliver 8 drops of urine into a test tube having an outside diameter of 19 millimeters. Next add 0.8 ml. of a 4.5 per cent solution of sulfosalicylic acid. The almost immediate appearance of a white, turbid precipitate or cloud indicates the presence of albumin. Now add 5 ml. of Benedict's solution and shake the tube well to mix the contents. Proceed as usual by boiling over the free flame for one or two minutes or by heating in a boiling water bath for five minutes. Allow the tube to cool in the air spontaneously. Interpret the result in the customary manner. This may be called the laboratory method.

The following modification should be useful to physicians, insurance medical examiners, visiting nurses, and others who do not wish to bother with reagent solutions. The test may be performed in the home, if desired. Deliver 5 drops of urine into a test tube having an outside diameter of 15 mm. and then add 10 drops of water. Drop in one sulfosalicylic acid reagent granule* and shake the contents gently for a second or two. After two minutes look for the turbidity described in the preceding paragraph. The granule does not dissolve, but it carries sufficient sulfosalicylic acid which is released to precipitate the protein. Proceed with the test for sugar by dropping in one effervescent sugar reagent tablet,† the active ingredients of which are cupric sulfate, sodium hydroxide, sodium bicarbonate, and citric acid. About fifteen seconds after the reaction has ceased, a typical copper reduction test will be obtained in the presence of sugar.

DISCUSSION

Sulfosalicylic acid is recognized as an excellent reagent for the precipitation of albumin in urine. The quantitative method described by Kingsbury, Clark, Williams, and Post‡ is widely used by life insurance companies, hospitals, and

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*Sulfosalicylic acid reagent granules No. 2 are manufactured and sold by Cargille Scientific Inc., New York, N. Y.

†Sugar reagent tablets are manufactured by Ames Company, Inc., Elkhart, Ind.

‡Kingsbury, F. B., Clark, C. P., Williams, G., and Post, A. L.: The Rapid Determination of Albumin in Urine, J. Lab. & Clin. Med. **11**: 981, 1925.

clinics. It was necessary in devising the method described herein to show only that the presence of sulfosalicylic said used in the test for albumin does not interfere with the test for sugar by Benedict's solution. The sugar reagent is sufficiently alkaline to neutralize the acid and it functions as though the sulfosalicylic acid were not present. Two series of urine containing 0.25, 0.5, 0.75, 1.0, and 1.5 per cent glucose were run, using in one case 0.8 ml. of water and in the other 0.8 ml. of a 4.5 per cent solution of sulfosalicylic acid plus 5 ml. of Benedict's solution. The two series were heated simultaneously in a boiling water bath for five minutes. Upon cooling, no difference in the results could be observed.

SUMMARY

A rapid method for detecting albumin and sugar in the same measured sample of urine is described.

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